INFLUENCE OF RED WINE GRAPE PHENOLIC EXTRACTS ON GLUCOSYLTRANSFERASE ACTIVITY, ACIDOGENICITY AND BIOFILM FORMATION OF *STREPTOCOCCUS MUTANS*

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy Food Science

by

Joanne Thimothe

January 2009

INFLUENCE OF RED WINE GRAPE PHENOLIC EXTRACTS ON GLUCOSYLTRANSFERASE ACTIVITY, ACIDOGENICITY AND BIOFILM FORMATION OF *STREPTOCOCCUS MUTANS*

Joanne Thimothe, Ph.D.

Cornell University 2009

Wine grapes are rich sources of potentially bioactive polyphenols. However, phenolic content is variable depending on grape variety, and may be modified during vinification. In this study, we investigated the influence of phenolic extracts from *Vitis vinifera* (Cabernet Franc and Pinot noir), *V. Labrusca* (Concord), and *Vitis* hybrids (Baco noir and Noiret™) grapes on several virulence properties of *Streptococcus mutans* a well-known cariogenic bacterium. Grape phenolic extracts were prepared from whole fruit (WF) and fermented pomace (FP). The anthocyanin and flavan-3-ol content were variable depending on variety and type of extract (WF vs. FP). Nevertheless, all extracts inhibited the activity of GTF B between 60-80% at concentrations as low as 31.2 μg/ml; GTF B synthesizes insoluble glucans, which are critical for the formation, bulk and structural integrity of biofilms. Furthermore, the glycolytic pH-drop by *S. mutans* cells was inhibited by the extracts without affecting bacterial viability, an effect that can be attributed to partial inhibition of F-ATPase by the extracts (30-65% inhibition at 125 μg/ml). In general, phenolic extracts from *V. vinifera* were more effective than those from *Vitis* hybrids. The biological activity of FP was either as effective as or significantly better than WF extracts. The most effective extracts were examined for their ability to disrupt biofilm development by *S. mutans* using a saliva-coated hydroxyapatite biofilm model. Biofilms were treated for 1 minute twice daily with phenolic extracts from FP of Cabernet Franc and Baco noir. Biofilms treated with the extracts (at 1 mg/ml) resulted in significantly lower amounts

of extracellular insoluble polysaccharides than those treated with vehicle control ($P<0.01$), which agrees well with the GTF B inhibition data. The reduction of insoluble polysaccharides production is highly relevant because they are the main components of the extracellular matrix of biofilms, and are associated with the pathogenesis of dental caries. Overall, the phenolic extracts, especially from pomace, are effective against specific virulence attributes of *S. mutans* and can disrupt the synthesis of extracellular polysaccharide matrix of biofilms. Fermented pomace could be a potential source for extraction of bioactive compounds for prevention or reduction of oral diseases, such as dental caries.

BIOGRAPHICAL SKETCH

Joanne Thimothe became fascinated with the ability to process agricultural products into wholesome, safe, and convenient food from her chemistry class in high school. Since then, she embarked on a journey that would eventually lead her to obtain her Bachelor of Science in Biology from Brooklyn College with a particular interest in microbiology. At Brooklyn College, she was enrolled in the Scholars Program and held several merit scholarships. In the Summer of 1999, after winning the Claire and Leonard Tow Summer Fellowship Award, she completed a Summer research program at Cornell University studying the pathogenicity of foodborne pathogens. About a year later, she joined the Cornell Food Science program where she obtained a Masters Degree in Food Science with a minor in Emerging Markets from the department of Applied Economics and Management. She is the main author of 3 currently published articles and a co-author of 3 more articles published in peer-reviewed papers.

Thimothe has also applied her food safety background to the food industry as she held close to 3 years of work experience. She has worked in the area of food safety and quality management with food companies in transition or working into making the transition toward implementing their HACCP (Hazard Analysis Critical Control Point) program. She has set up a total quality management system for a company to receive grant of inspection from the U.S. Department of Agriculture (USDA). Her food safety experience has allowed her to work with regulatory agencies such as the Food and Drug Administration, the New York City Department of Health Services, and the USDA.

A dream that started in high school has turned into reality. Therefore, I will dedicate this dissertation to the women who helped make this dream possible, Ducile Severe (in memoriam), Julida Clarilus, and Kettly Caldwell. These women have selflessly sacrificed their time, given their money, and opened their home to make it possible for me to get to my dream. From the first day, they have embraced my dream. They had confidence in me even when they had no idea where I was heading. I am indebted to their generosity, patience, and words of encouragements. .

ACKNOWLEDGMENTS

I am also very fortunate to have people who set the pace and guided me throughout my journey. I thank members of my current committee for their time, patience, and continued support:

Dr. Olga Padilla-Zakour,

Dr. Hyun (Michel) Koo (University of Rochester),

Dr. Kathryn J. Boor,

Dr. David BenDaniel.

This project was supported by the Field of Food Science and Technology SUNY fellowship program and the National Research Initiative Grant No. 2006-35200-16589 from the USDA Cooperative State Research, Education, and Extension Service – Bioactive Food Components for Optimal Health (31.0) program.

Travel grants and Scholarships were provided by the Western New York Institute of Food Technologists section and the Department of Food Science.

I thank both Cornell University and the Eastman Dental Center at the University of Rochester School of Medicine for providing access to a wealth of information from their faculty, support staff, and libraries.

TABLE OF CONTENTS

BIOGRAPHICAL SKETCH iii

DEDICATION iv

ACKNOWLEDGMENTS v

TABLE OF CONTENTS vi

LISTS OF FIGURES ix

LISTS OF TABLES x

LIST OF ABBREVIATION xi

CHAPTER 1

INTRODUCTION 1

1.1 Overview 1

1.2 Grapes: Background 1

1.2.1 General composition of grapes 3

1.2.1.1 Flavonoids 4

1.2.1.2 Non-Flavonoids 6

1.2.2 Polyphenol distribution in grapes 8

1.2.2.1 Polyphenols in seeds 8

1.2.2.2 Polyphenols in skins 9

1.2.2.3 Polyphenols in stems 9

1.2.3 Grape Pomace 10

1.2.4 Phenolic composition of grapes based on varieties and methods of processing 11

1.2.4.1 Grape Variety 12

1.2.4.2 Processing methods and other factors 14

1.2.5 Biological activities of grape 15

1.3 Dental caries disease: Background 17

1.3.1 Dental caries - A dietobacterial biofilm related disease 17
1.3.2 Virulence factors 20
1.3.2.1 Glucan synthesis 20
1.3.2.2 Acid Production and Acid Tolerance 21
1.3.3 Chemotherapeutic methods to control dental caries 22
1.3.3.1 Fluoride 22
1.3.3.2 Antimicrobials 23
1.3.4 Natural products as an alternative for novel anti-caries therapy 24
1.4 Hypotheses 25
1.5 Objectives 26
CHAPTER 2
MATERIALS & METHODS 27
2.1 Grape procesing 27
2.2 Grape Extracts 27
2.3 Determination of phenolic composition of the grape extracts 30
2.4 Determination of the biological activity of the grape extracts 31
2.4.1 Bacterial strains 31
2.4.2 Glucosyltransferases (GTF) assays 31
2.4.3 F-ATPase and glycolytic pH drop assays 33
2.4.4 Antimicrobial assays 33
2.4.5 Biofilm assays 34
2.4.5.1 Biofilm preparation and treatments 34
2.4.5.2 Biofilm analyses 34
2.5 Statistical analyses 36
CHAPTER 3
RESULTS AND DISCUSSION 38

3.1 Selection of grape extracts 38
3.2 Contribution of major phenolic compounds 39
3.3 Influence of grape extracts on GTF activities 41
3.4 Influence of grape extracts on *S. mutans* acidogenicity 46
3.5 Effects of the grape extracts on polysaccharide composition in biofilms 51

CHAPTER 4

SUMMARY AND FUTURE RESEARCH 55

APPENDIX A: GTF B solution assay 60
APPENDIX B: GTF B solution for Concord grapes variety 65
APPENDIX C: GTF B surface 67
APPENDIX D: F-ATPase data 69

REFERENCES 74

LIST OF FIGURES

1.1 Structure of flavonoids 7

1.2 Crushed grapes during fermentation 10

1.3 Scanning electron micrograph of oral bacteria enmeshed in EPS matrix formed *in situ* on hydroxyapatite surface 18

1.4 The 3 major steps found during biofilm formation 19

2.1. Diagram for the processing of Whole Fruit and Fermented pomace prior to biological testing. 29

2.2 Summary diagram of biological analyses. 32

2.3 Saliva-coated hydroxyapatite (sHA) biofilm model 35

3.1. Schematic of the grape varieties and methods of processing used in this study. 40

3.2 Total Phenolic and Anthocyanin content of grapes 41

3.3 Influence of grape extracts on the activity of GTF B in solution 43

3.4 Influence of grape extracts on the activity of GTF B on surface 45

3.5 Influence of grape extracts on glycolytic pH drop assay of *S. mutans* UA 159 in suspension 48

3.6 Effect of selected grape extracts on F-ATPase activity 49

LIST OF TABLES

1.1 Production of selected grapes in New York State (2006-2007) 3

1.2 Polyphenolic composition of grapes 5

1.3 Current and potential utilization of pomace 11

1.4 Phenolic composition found in pomace, wine grapes, fresh grapes, and grape juice 13

1.5 Biological activities of grape polyphenols 16

3.1 Total Phenolic, Anthocyanin, and Flavan-3-ol Content of Freeze-dried Grape Phenolic Extracts. 42

3.2. Polysaccharides content in the biofilms after treatments with grape extracts. 53

LIST OF ABBREVIATIONS

AN	Anthocyanins
BNFP	Baco noir fermented pomace
Cab. Franc	Cabernet Franc
Cat Eq	Catechin equivalents
CFFP	Cabernet Franc fermented pomace
GTF	Glucosyltransferase
FP	Fermented pomace
GAE	Gallic acid equivalents
INS	Insoluble
IPS	Intracellular Iodophilic Polysaccharides
Mal Eq	Malvidin-3-glucoside equivalents
PNFP	Pinot noir fermented pomace
SOL	Soluble
TP	Total Phenols
WF	Whole Fruit

CHAPTER 1

INTRODUCTION

1.1 Overview

Grapes are one of the world's most produced fruit crops with an annual production of roughly 66 million metric tons in 2005 (FAOSTAT-FAO). More than 80% of the grapes produced are used for making wine. Wine grapes have been recognized as a rich source of potentially bioactive polyphenols, which has led to an increased interest in their chemical composition and biological properties, including antimicrobial activity. *Streptococcus mutans* is the main microbial culprit involved with the development of dental caries in humans, a ubiquitous oral disease that continues to plague the majority of the world's population. This bacterium has the ability to adhere firmly to and colonize the tooth surface, and metabolize dietary carbohydrates provided by the human host. This metabolism results in extracellular polymers which form the matrix of dental biofilm (known as plaque) and organic acids which cause tooth enamel to dissolve, ultimately resulting in decay. The grape and its constituents may interact with oral bacteria (e.g. *S. mutans*) and potentially disrupt their expression of virulence, leading to novel alternatives to prevent or reduce dental caries.

1.2 Grapes: Background

The origins of grape cultivation date back to 3500-2300 BC in the Jordan Valley (Zohary and Hofp 1993). It is not known how much the discovery of wine contributed to the domestication of grapes but historically, wine was believed to be a divine elixir (McGovern 2004). For the next 2000 years, grape cultivation spread throughout Eurasia and Northern Africa (De Candolle 1886; Stager 1985). As the Roman Empire conquered new territories and expanded their trade routes, they also

left behind a culture of grape domestication (This and others 2006). Many of the currently available grape varieties bear Latin names given to them by the Romans. After the Romans, European missionaries from the Catholic Church carried with them grape seeds and cuttings from their places of origin to America and other parts of world (This and others 2006).

Grape cultivars belong to the botanical family Vitaceae that includes 12 to 14 genera (Burns 2002). More than 9600 varieties of grapevines exist around the world (Galet 2000). The *Vitis* genus has the greatest agronomic importance and is generally grouped into wine grapes, table grapes, and raisins (This and others 2006). *Vitis vinifera* are sensitive to cold winters, short growing seasons, and lack resistance to local grape diseases (Reisch and others 1993a). Few European varieties of *V. vinifera* have been grown successfully in New York State. Still, among the *V. vinifera* varieties available in New York State, Pinot noir and Cabernet Franc are two of the most successfully cultivated. Non-vinifera American varieties are more resistant to the climate and grape diseases than the *V. vinifera.* Therefore, several interspecific hybrids, like Baco noir, were developed, combining European varieties and American varieties like *Vitis labrusca* (Polleyfeys and Bousquet 2003; Reisch and others 1993a, b).

V. labrusca includes table grapes such as Concord, Niagara, and Catawba used for fresh consumption and in the production of juice, preserves, and wines. In New York, Concord grapes are one of the most cultivated grape varieties (Table 1.1) with 102, 924 tons produced in 2006 and 131,000 tons in 2007 (NASS 2007; 2008).

Table 1.1 Production of selected grapes in New York State (2006-2007)

		2006[1] (in tons)	**2007[2] (in tons)**
American varieties	Catawba	5,674	4,930
	Concord	102,924	131,000
	Niagara	18,616	21,000
Hybrids	Baco noir	692	430
	Noiret™	70	n/a
Vitis vinifera	Cabernet Franc	1,430	n/a
	Pinot noir	1,014	n/a

[1] NASS, New York Fruit and tree and vineyard survey; [2] NASS, Fruit report, July 2008

1.2.1 General composition of grapes

Grapes contain 80-90% juice, 5-12% skins, 2-6% stems, and 0-5% seeds (Amerine and Joslyn 1967). They also contain water, sugar (glucose, fructose), organic acids (tartaric, malic, shikimic), and phenolic compounds (Falchi and others 2006). In recent years, an increasing emphasis has been placed on the health benefits of phenolic substances, polyphenols, in the diet. Close to 90% of the total extractable phenols in grapes are concentrated in the seeds and skins (Amerine and Joslyn 1967).

The major phenolic compound classes in grapes are divided into two categories: 1) the flavonoids, which include anthocyanins, flavonols, flavan-3-ols, tannins, and flavanonols, and 2) non-flavonoids, including phenolic acid cinnamates and derivatives, benzene derivatives, tyrosol, and volatile phenols (Table 1.2). Flavonoids are structured as a (C_6 –C_3- C_6) basic skeleton (Figure 1.1), but they differ

in the number of substituent hydroxyl groups, degree of unsaturation, and degree of oxidation of the three-carbon segment (Spanos and Wrolstad 1990).

1.2.1.1 Flavonoids

Anthocyanins are water soluble and responsible for the red, purple, blue, and orange colors of fruits, vegetables, flowers, and grape skins (Ju and Howard 2003). In some grape cultivars, anthocyanins are also accumulated in the pulp (Boulton and others 1995).

Anthocyanins are a major component of red wine color and contribute to the organoleptic and chemical properties of grapes and wines (Mazza and Miniati, 1993). The six common aglycones are delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. Anthocyanin pigments are glycosylated generally with a glucose moiety at position 3 (Manach and others 2004). Acetyl and cinnamoyl residues are sometimes attached to the glycosyl group (Ribereau-Gayon1982; Mazza and Miniati 1993).

Flavonols are flavonoids with an unsaturated 3-C chain with a double bond between C-2 and C-3, and the presence of a hydroxyl group in the 3-position (Macheix and others 1990) (Figure 1.1). The flavonols occur mostly as glycosides where glucose, galactose, rahmnose, arabinose, xylose, and glucoronic acid can become attached to the flavonol structure at position 3 (Macheix and others 1990). Quercetin and kaempferol 3-rutinosides are the most common diglycosides (Macheix and others 1990). The flavan-3-ols are present in grapes either as monomers or linked to each other to form procyanidins or proanthocyanidins, also called condensed tannins (Figure 1.1). Some of the flavan-3-ols monomers are: (+) catechin, (-) epicatechin, (-) epigallocatechin, epicatechin gallate; catechin and epicatechin are the most common

Table 1.2 Polyphenolic composition of grapes

Phenolic	Classes	Compounds	References
Flavonoids	Anthocyanins	delphinidin, cyanidin, petunidin, pelargonidin, peonidin, malvidin	Ribereau-Gayon 1982; Mazza and Miniati 1993
	Flavonols	quercetin, kaempherol, myricetin, isorhamnetin	Singleton, 1992; Macheix and others 1990
	flavan-3-ols (monomers)	(+) catechin, (-) epicatechin, (+) gallocatechin, (-) epigallocathechin,	Macheix and others 1990
	(dimers, polymers)	epicatechin-3-O-gallate, procyanidins B1, B2, B3, B4, C1, C2, made of flavan-3,4-diols and flavan-3-ols	
Non-flavonoids	Phenolic acids: Benzoic acids and derivatives	gallic acid, cis-and trans coutaric acids, protocatechuic acid, syringic acid, vanillic acid	Lu and Foo, 1999; Kallithraka and others 2006
	Phenolic acids: Hydroxycinnamic acid and derivatives	caffeic acid, p-coumaric, ferulic acid, chlorogenic acid, caftaric acid, fertaric acid	Wen and others, 2003; Macheix and others, 1990
	Stillbenes	Resveratrol	Waterhouse, 2002

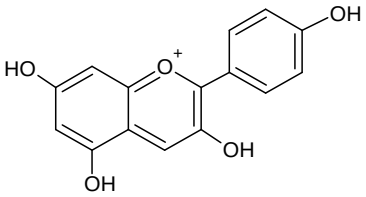
Flavan-3-ols found in grapes (Alonso-Borbalán and others 2003; Macheix and others 1990). Procyanidins are polymers (dimers through decamers) of flavan-3-ols repeated molecules. Procyanidins are more abundant than simple flavan-3-ols and dimers (Macheix and others 1990). Polymers have a molecular weight between 500 to 3000

(Robichaud and Noble 1990). The main type of linkage found in grapes is the B-type linkage where the monomeric structures are linked from C4 to C8 or to C6 (Sarni-Manchado 1999). Four B-type dimers are usually observed: B1, B2, B3, B4 (Macheix and others 1990). B1 is the most common from grape skins. The average percentage of procyanidins B1 from grape skin has been reported at 34% (Macheix and others 1990).

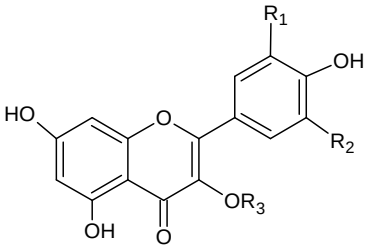
Proanthocyanidins are polymers of polyhydroxy flavan-3-ol such as (+)-catechin and (-)-epicatechin (Porter 1986; Burns and others 2000). They derive from the condensation of flavan-3-ol units (Watherhouse 2002). Proanthocyanidins can be composed of procyanidins and/or prodelphinidins depending on the presence or absence of an H or OH in R_4 position as shown in Figure 1.1 (Torres and others 2002). Proanthocyanidins are also responsible for the organoleptic properties of wine such as color, astringency, bitterness and contribute to haze formation and interactions with proteins (Haslam 1980; Cheynier and Silva 1991; Ricardo da Silva and others 1991).

1.2.1.2 Non-Flavonoids

Non-flavonoid phenols are derivatives of hydroxycinnamic and hydroxybenzoic acids such as coutaric, caftaric, gallic, ellagic, and other low molecular weight phenolics (Singleton 1992). These compounds are particularly abundant in white wine and are present in the pulp. Hydroxycinnamic derivatives are the major non-flavonoids found in wines (Singleton and others 1985) and contribute to bitterness (Noble 1990). Non-flavonoids also include stillbenes and resveratrol. Hydroxylated stillbenes are found as glycosides in grapes (Waterhouse 2002).

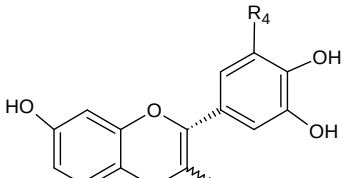


Anthocyanins



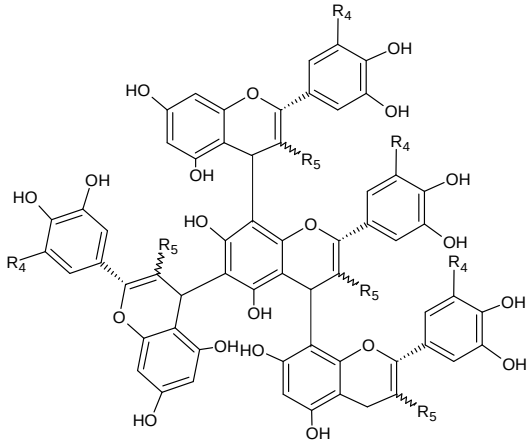
R_1= R_2= H, R_3=H: Kaempferol
R_1= OH, R_2= H, R_3=H: quercetin
R_1= OH, R_2= OH, R_3=H: myricetin
R_3= sacccharide: flavonol glycosides

Flavonols



R_4= H: catechins, OH: gallocatechins

Flavan-3-ols



R_4 = H: procyanidins, OH: prodelphinidins
R_5 = OH (putative polymerization position)

Proanthocyanidins (flavan-3-ols polymers)

Figure 1.1. Structure of flavonoids. (Torres and others 2002; Manach and others 2004; Flamini and others 2003).

1.2.2 Polyphenol distribution in grapes

Grape seeds, stems and skins are a rich source of polyphenols with seeds and skins having the most flavonoids (Souquet and others 2000; Diplock and others 1998; Cheynier and Rigaud 1986; Su and Singleton 1969; Alonso-Borbalán and others 2003). The seeds have the highest polyphenol content by weight at 5-8% (Amerine and Joslyn 1967). Polyphenols in grape seeds are mostly flavonoids and have higher concentrations of catechins and procyanidins (mainly B2) than skins and pulp (Peña-Niera and others 2004; Jayaprakasha and others 2001; Kammerer and others 2004; Souquet 1996; Fuleki and Ricardo da Silva 1997).

1.2.2.1 Polyphenols in seeds

Procyanidins are the only type of proanthocyanidins that can be found in grape seeds (Figure 1.1). HPLC following thyolysis degradation releases only catechin, epicatechin, and epicatechin-3-*O*-gallate which indicates that grape seed tannins are procyanidins and not prodelphinidins (Prieur and others 1994; Labarbe and others 1999). Fuleki and Ricardo da Silva (1997) also identified flavan-3-ols from grape seeds as 1) monomers: (+)-catechin, (-)-epicatechin, (-)-epicatechin 3-O-gallate; 2) dimer procyanidins; 3) dimers esterified with gallic acid B1-3-O-gallate, B2-3-O-gallate; 4) trimer procyanidins C1, C2, T2,T3, T4, T5, and T6; 5) trimers esterified with gallic acid; and 6) tetramer procyanidins.

The procyanidins from grape seeds are all esters of gallic acid (Lee and Jaworski 1990). Close to 55% of grape seed procyanidins are of the polymeric type with a degree of polymerization greater than 5 (Prieur and others 1994). The mean degree of polymerization for proanthocyanidins (condensed tannins) from grape seeds

(cv. Cabernet Franc) ranges from 4.7 to 17.4 and for those isolated from skins, ranges between 9.3 to 73.8 (Labarbe and others 1999; Souquet and others 2000).

1.2.2.2 Polyphenols in skins

Grape skins, rich in polyphenols, can be transferred to wine (Gagne and others 2006) and contribute to wine color. Anthocyanin content increases at maturity and contributes to color change (Burns and others 2002). In addition to anthocyanins, other phenolic compounds such as procyanidins can also form a complex with anthocyanins that affect wine color (Glories 1988). Malvidin 3-*O*-glucosides are the major anthocyanins in grape skins in addition to delphinidin, cyanidin, petunidin, peonidin, and some acids (Burns and others 2002). Total anthocyanins from grape skins of red grape varieties of *V. vinifera* range from 81.3 to 144.6 mg/100g of fresh weight while total polyphenols were determined to be between 182.7 to 327.9 mg/100g of fresh weight (Falchi and others 2006).

Grape skin tannins are more polymerized than seed tannins with a degree of polymerization ranging from 3 to 80 (Souquet and others 1996) while the polymerization of seeds can range from 2 to 15 (Prieur and others 1994). About 90% of skin proanthocyanidins have a degree of polymerization greater than 10 (Souquet and others 1996). Phenolic acids are found in the skins in a much lower quantity than anthocyanins (Zafrilla and others 2003).

1.2.2.3 Polyphenols in stems

Grape stems can also contribute to phenolic composition in wine. Wine made with whole grapes has more phenolic compounds than varieties made with destemmed

grapes (Souquet and others 2000). About 8% of stems are still present on grape berries during wine production (Souquet and others 2000). Grape stems contain polyphenolic compounds that include phenolic acids, flavonols, and flavanonols, as well as tannins in much lower quantity than in skins. The level of tannins in stems (221.3 mg tannins/kg crop) is considerably below that of skins (1605.0 mg tannins/kg crop) and seeds (2323.5 mg tannins/kg crop) (Souquet and others 2000). Like grape skins, stem tannins contain prodelphinidins. Stems showed only trace amounts of epicatechin, kaempherol 3-glucoside and myricetin 3-glucoside (Souquet and others 2000).

Figure 1.2. Crushed grapes during fermentation. After extraction of the juice, the leftover skin and seed is then called pomace.

1.2.3 Grape Pomace

Pomace is a waste product obtained from crushing grapes to produce wine (Figure 1.2). About 13-20% of the grapes by weight is turned into pomace (Torres and Bobet 2001; Torres and others 2002). Pomace consists mostly of skins (41.5%),

seeds (22%), and stems (15.1%) (Amerine and Joslyn 1967). Its potential uses and applications as a low cost and readily available source for the extraction of nutritional supplement or in the extraction of phytochemicals are summarized in Table 1.3. Pomace contains many phenolic compounds also found in grapes because of poor extraction during winemaking (Lu and Foo 1999; Kammerer and others 2004). Pomace is one of the main sources of anthocyanins (Schieber and others 2001).

Table 1.3 Current and potential utilization of grape pomace

Uses	**References**
Citric acid production	Hang and Woodams 1986
Dietary supplements	González-Paramás and others 2004; Kammerer and others 2004
Energy production	Blasi and others 1997
Fertilizer	Manios 2004
Grappa (distilled spirit)	Hang and Woodams 2008
Grape seed oil	El-Shami and others 1992
Tartaric acid	Nurgel and Canbas 1998; Braga and others 2002

1.2.4 Phenolic composition of grapes based on varieties and methods of processing

The phenolic composition of grapes varies with species, variety, degree of maturation and environmental factors such as climate (Jackson and Lombard 1993;

Fuleki and Ricardo-da-Silva 1997; Basha and others 2004) (Table 1.4). Particular species and varieties have a major influence on the amount and composition of procyanidins in grape seeds (Fuleki and Ricardo-da-Silva 1997). The amount of procyanidins B2 reported by Fuleki and Ricardo-da-Silva (1997) from 17 varieties varied from 9 to 106 mg/100g.

1.2.4.1 Grape Variety

In general, red grape varieties have higher phenolic content, especially anthocyanins than their white counterparts (Vinson and Hontz 1995; Alonso- Borbalan and others 2003; Kammerer and others 2004). Total phenolic content of red wine extracts was found to be between 2,138 and 24,980 mg/L of gallic acid equivalents (Papadopoulou and others 2005) while that of white wine was between 1,210 and 5,320 mg/L of gallic acid equivalents. The observed difference in total phenols between red and white wine is due to minimal contact with the skin, stems, and seeds of the grapes during the processing of white wine (Singleton 1992).

In white wine, there is limited extraction and oxidation of flavonoids since the skin is removed during vinification. Therefore, the phenolic compounds usually obtained from the skins in red wines processing are not available. The catechin level found in a red wine variety (548.77 mg/L), Pinot noir, was higher than the catechin level found in the white wine variety, Chardonnay (371.9 mg/L) (Landrault and others 2001). Phenolic acids from the pulp are the primary phenolic compounds in white wines (Sims 1994). Hydroxycinnamic acids, especially caftaric acid, are the major phenolics (Cheynier and others 1998).

Table 1.4 Phenolic composition found in pomace, wine grapes, fresh grapes, and grape juice

Grape color	Samples	Grape varieties	Total phenols[1] (mg GAE)	Total anthocyanins[1] (mg mal eq)	References
Red	Skin	Cabernet Franc	462– 1009	796-1072.4	Mazza and others 1999
		Pinot noir	806 – 1074	754 - 806.7	Mazza and others 1999
	Wine	Pinot noir	188-193	-	Chamkha and others 2003
		Pinot noir	2329		Landrault and others 2001
		Merlot	1783		Landrault and others 2001
		Red wine	1732	400	Waterhouse 2002
White	Wine	Chardonnay	176-195	-	Chamkha and others 2003
		Semillon Sweet	724	-	Landrault and others 2001
		Average dry white	312.7 – 516.1	-	Landrault and others 2001
		Average table white	209.5 – 285.5	-	Waterhouse 2002
Red Table grapes	Grapes	Red Globe	225.4	115.3	Cantos and others 2002
		Flame	353.2 -369.2	150.7	Cantos and others 2002
		Napoleon	129.6 - 142.2	75.7	Cantos and others 2002
	Juice	Concord	23-195	10.64-28.5	Fuleki and Ricardo-da-Silva 2003

[1]Contents are expressed in mg GAE/kg of grapes or mg GAE/L of wine. Abbreviations GAE: gallic acid equivalents; Mal equ: malvidin-3-glucoside equivalents.

As opposed to wine grapes, table grapes are mainly used for fresh consumption. Although some table grapes belong to the same genus as wine grapes, they differ in their chemical components. Wine grapes contain higher total phenolic content than table grapes (Table 1.4). The main anthocyanin in many table grapes was reported to be peonidin-3-glucoside (Cantos and others 2002) whereas malvidin 3-glucoside is the most common anthocyanin in wine grapes (Mazza 1995).

1.2.4.2 Processing methods and other factors

Degree of maturation also affects phenolic content. The amount of anthocyanins in grapes increased at maturation while the amount of catechins and procyanidins decreased (Pérez-Magariño and González-San José 2004; Shi and others 2003). At a similar degree of maturation, the phenolic content in grapes still differed when the grapes were harvested from two different years or two different season (Revilla and others 1995; Mazza and others 1999). Catechin levels for red wine of southern France from vintages of 1999, 1997, 1995-1991 were at 219.13, 153.99, and 88.28 mg/L respectively (Landrault and others 2001).

Processing conditions and temperature affected the extraction of phenolics from grapes (Blanco and others 1998). Mazza and others (1999) found that total phenolics and flavonols increased during alcohol fermentation. There were quantitative differences in flavanols depending on the method of processing pomace both within the same variety and among the different varieties (González-Paramás and others 2004). Maceration increased the availability of polyphenols (Fuleki and Ricardo-da-Silva 2003).

Temperature conditions during maceration affected extractability of the phenolics (Koyama and others 2007). Total phenolics expressed as gallic acid were

significantly higher in hot pressed Concord grape juice (195.34 mg/L) when compared to cold pressed (23.06 mg/L) (Fuleki and Ricardo-da-Silva 2003). Elevated temperature increased extraction of phenolics from the skins, especially anthocyanins. On the other hand, the amount of catechin and epicatechin was greater in cold pressed juice left for 18h than in hot pressed juice left for 30, 60, and 90 minutes (Fuleki and Ricardo-da-Silva 2003).

1.2.5 Biological activities of grape

The presence of flavonoids in wine and grape products has been associated with their biological activities, including antimicrobial activities (Table 1.5). Daroch and others (2001) found that all of the 16 red wines studied had antibacterial activity against *H. pylori* and determined that the active compound was resveratrol. Quercetin, (+)-catechin, (-)-epicatechin, trans-resveratrol, gallic acid and protocatechuic acid have been shown to inhibit activity against mutagens using *Salmonella typhimurium* strain TA102 (Stagos and others 2006). Phenolic acids from red and white wines have shown inhibitory activities against strains of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Papadopoulou and others 2005).

The biological activities of grape polyphenols have been found to be dependent on which part of the grape the polyphenols were extracted from. Ribier grape pulp was not inhibitory whereas the skin and seeds had strong antilisterial activity (Rhodes and others 2006). The microbiological benefits of grapes and their by-products have been extensively studied on a variety of microorganisms. However, few studies have looked at the biological activities of grape phytochemicals and their relationship with the microorganisms associated with dental caries.

Table 1.5 Biological activities of grape polyphenols

Phenolic compounds	Effects	Source	References
Flavanols	Antioxidant	Wine, grape pomace, grape seeds	González-Paramás 2004
Catechin monomers, procyanidin oligomers, flavonol derivatives	Antioxidant	Parellada grape (*Vitis vinifera)* pomace	Torres and others 2002
Anthocyanins and seed derived polymer	Antilisterial	Ribier grape juice, skin, extracts (*V. vinifera*)	Rhodes and others 2006
Resveratrol (stillbenes)	Antimicrobial properties (*Helicobacter pylori)*	Chilean red wines	Daroch and others 2001
Proanthocyanidin	Antioxidant activities and potential agent against periodontal diseases	Grape seed extracts	Houde and others 2006
Procyanidin B5-3'-gallate	Anti-tumor	Grape seed extracts (*V. vinifera)*	Zhao and others 1999
Quercetin, catechin, epicatechin, trans-resveratrol, gallic acid and protocatechuic acid	Inhibition of mutagens in Salmonella typhimurium	Grape extracts (*Vitis vinifera)*	Stagos and others 2006

1.3 Dental caries disease: Background[1]

Dental caries is the single most prevalent and costly infectious oral disease (NIH 2001; Marsh 2003). This ubiquitous disease afflicts the majority of the adult population in the United States and continues to be a major reason for attendance at emergency rooms and a leading cause of absence from work (US Department of Health and Human Services 2000). The public in the United States spends close to $40 billion to treat the ravages of this disease and its consequences. This does not include the billions expended in oral hygiene products aimed at prevention of the disease and the removal of dental plaque. Thus, dental caries places enormous health and economic burdens on Americans most often on those least able to bear them (NIH 2001; Bowen 2002).

1.3.1 Dental caries - A dietobacterial biofilm related disease

Dental caries results from the interaction of specific bacteria with constituents of the diet within a dental biofilm known as plaque (Bowen 2002). *Streptococcus mutans* is generally regarded as the primary microbial culprit although additional microorganisms may be involved (Hamada and Slade 1980; Loesche 1986; Beighton 2005); this bacterium produces extracellular polysaccharides (EPS) in dental biofilms through glucosyltransferases (GTFs) and fructosyltransferase (FTF), and is acidogenic and acid-tolerant. Sucrose is considered to be the "arch criminal" from the dietary aspect because it is fermentable, and also serves as a substrate for synthesis of EPS and intracellular polysaccharides (IPS) in dental biofilm (Bowen 2002). However, it is important to emphasize that additional sugars and starch can certainly contribute to the pathogenesis (Bowen and others 1980; Firestone and others 1982).

[1] Reproduced in part with permission from Dr. Hyun Koo. The research related to the dental portion of the research was conducted in Dr. Koo's lab at the University of Rochester Eastman Dental Center.

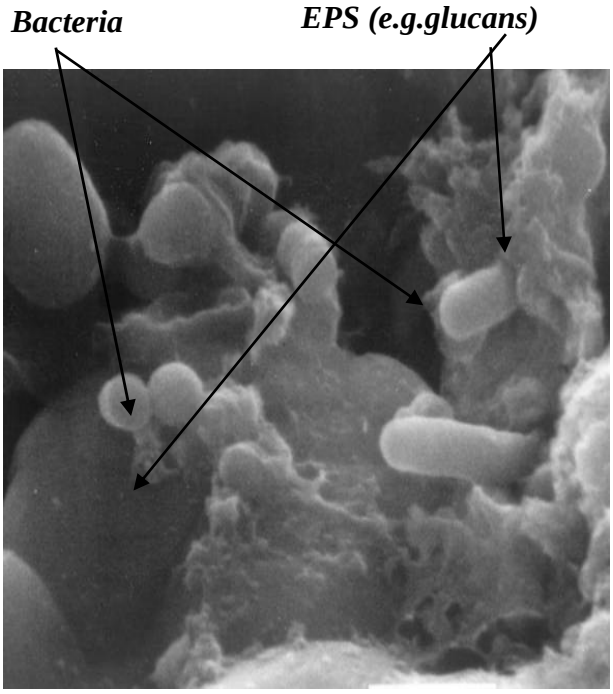


Figure 1.3. Scanning electron micrograph of oral bacteria enmeshed in EPS matrix formed *in situ* on hydroxyapatite surface (Adapted from Vacca-Smith and Bowen, 2000).

The first clinical evidence of the bacterial dietary interaction is the appearance of dental plaque. Dental plaque is a biofilm; it is composed of bacteria and salivary constituents enmeshed in a polysaccharide matrix and is tightly adhered to the tooth surface (Figure 1.3) (Critchley 1969; Hotz and others 1972; Holt 1975); up to 40% of the dry weight of dental biofilm is composed of polysaccharides (Emilson and others 1984; Bowen 2002; Paes Leme and others 2006).

If dental biofilm is allowed to remain on tooth surfaces with a frequent consumption of a high carbohydrate diet (especially sucrose), *S. mutans* and other acidogenic bacteria will metabolize the sugars to organic acids and polysaccharides

(Bowen 1999**)**. The resulting low pH environment favors the growth of cariogenic aciduric streptococci (*S. mutans* for example), and the elevated amounts of EPS

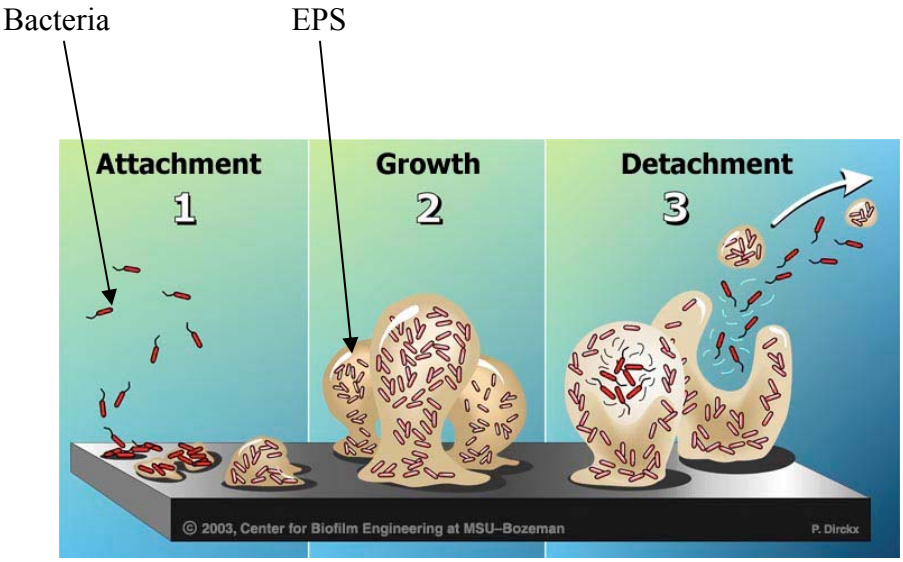


Figure 1.4 The 3 major steps found during biofilm formation.

promote biochemical and structural changes in the matrix of the biofilm (Schilling and Bowen 1992**)**. The persistence of this acidic condition triggers a shift in the biofilm community towards dominance by acidogenic and aciduric bacteria, such as *S. mutans*, because of their ability to survive low pH values (Marsh 2003). The low pH microenvironment in the biofilms matrix results in dissolution of enamel of tooth surface. Clearly, EPS are essential for the formation and establishment of cariogenic biofilms (Tanzer and others 1985; Yamashita and others 1993; Schilling and Bowen, 1992; Marsh 2003; Kuramitsu 2003). Furthermore, it has been demonstrated to augment the cariogenicity of dental biofilms in clinical studies (Cury and others 1997;

2000; Mattos-Graner and others 2000; Nobre do Santos and others 2002; Aires and others 2006). Therefore, biofilm control strategies based on disruption of EPS offer an attractive and alternative approach to the use of broad spectrum antibiotics like chlorohexidine (Xavier and others 2005; Lewis 2001).

1.3.2 Virulence factors

1.3.2.1 Glucan synthesis

GTF enzymes have been recognized as virulence factors in the pathogenesis of dental caries (Yamashita and others 1993). *S. mutans* produces at least three distinct GTFs: GTF B, C, and D (Loesche 1986; Hanada and Kuramitsu 1988; Yamashita and others 1993). The GTFs synthesize water insoluble α(1,3)-linked glucans (GTF B and C) and water soluble α(1,6)-linked glucans (GTF C and D). GTF B and C have been shown to be essential for the expression of virulence in rat caries models (Yamashita and others 1993).

Although both GTF B and C play an essential role in the expression of virulence, they differ in their chemical structure. The primary amino acid sequences of the streptococcal GTF enzymes are highly homologous (Banas and Vickerman 2003). The secondary structures contain a circularly permuted $(alpha/beta)_8$-barrel motif (MacGregor and others 1996). The GTF enzymes have approximately a signal peptide of 38 amino acids at the amino terminus followed by a variable domain of about 200 amino acids distinctive for each enzyme (Russell 1994). GTF B differs from GTF C by their C-terminus. The C-terminus contains a series of repeat motifs of amino acids that vary for each enzyme. GTF B contains 1475 amino acids (Shiroza and others 1987) while GTF C contains 1375 amino acids with a small hydrophobic domain in the direct repeat units at the C-terminus (Ueda and others 1988). The structural difference of the C-terminus noted from GTF B and GTF C might explain

the affinity of GTF B to bacterial surfaces and that of GTF C to apatitic surfaces (Vacca-Smith and Bowen, 1998).

GTF can be found in whole human saliva (solution) and in the salivary pellicle on the tooth surface. GTFs secreted by *S. mutans* bind avidly in the presence of saliva to the tooth surface and are highly active and when exposed to sucrose; a layer of polysaccharides is formed rapidly on the surface (Rolla and others 1983; Schilling and Bowen, 1988; 1992; Rozen and others 2004). The polysaccharides on the surface provide an avid site for colonization by bacteria. GTFs also adhere to bacterial surfaces of both GTFs-producing and non-GTFs producing bacteria, and furthermore adhere to surfaces of bacteria that do not make enzyme, thereby converting them into *de facto* glucan producers (McCabe and Donkersloot, 1977; Vacca-Smith and Bowen, 1998). Thus, glucans promote the accumulation of microorganisms on the tooth surface, and contribute to the bulk of the biofilms (Schilling and Bowen 1992). Figure 1.4 shows the major steps of biofilm formation process.

1.3.2.2 Acid Production and Acid Tolerance

S. mutans survives and carries out glycolysis at low pH values attained within the matrix of the biofilms which results in demineralization of the adjacent dental enamel (Belli and Marquis 1991; Bowen 2002). The actual pH to ensure demineralization is 5.5 (Stephan 1944). The ability of *S. mutans* to adapt to acidic environments is due in part by the regulation of the proton-translocating F-ATPase that helps *S. mutans* maintain intracellular pH more alkaline than the extracellular pH (Bellli and Marquis 1991; Sturr and Marquis 1992). ATPase inhibitors increase proton permeability of the membrane (Bender and others 1986) and dissipate the pH gradient. With constant exposure of the mouth to an acidic environment, aciduric and

acidogenic microorganisms will prevail and predispose the teeth to dental caries (Marsh 2003).

1.3.3 Chemotherapeutic methods to control dental caries

S. mutans is a key contributor in the plaque formation and in the pathogenesis of dental caries. Anti-caries products aimed at controlling acid production and tolerance, glucans synthesis and at reducing the levels of *S. mutans* have been investigated. The most documented strategies are discussed below. They include: fluoride, antimicrobials such as chlorhexidine and triclosan.

1.3.3.1 Fluoride

The benefits of fluoride have been demonstrated since the beginning of the 19th century (Van Loveren 2001). Fluoride is by far the most proven anti-caries agent known (Bowen 2002) and has been widely used in products aimed at controlling dental caries. Fluoride is found in products such as toothpaste, mouth rinses, dental varnishes, water, and salt (Brambilla 2001). Fluoride promotes remineralization and can reduce risk of caries (Sheiham 1991; Featherstone 2006). The effectiveness of fluoride depends on the pre-existing pH in the mouth and the pre-existing concentration of fluoride (Marquis 1990; Clarkson and others 1986). Only 0.1mM of fluoride at pH 4.0 was required to inhibit glycolysis whereas 10 mM was required at pH 6.0 (Marquis 1990).

Fluoride was found to be most effective at reducing acid tolerance of glycolysis of intact cells of *S. mutans* (Belli and others 1995). The ionized form of fluoride can penetrate bacterial membranes under acidic conditions (Marsh 2003). Once inside the cell, the intracellular H^+ F^- dissociates and F^- binds to key enzymes

like F-ATPase that regulate intracellular pH and proteins important for glycolysis while the H^+ lower the pH in the cytoplasm (Marsh 2003; Marquis and others 2003). Fluoride acts as inhibitor for F-ATPase in presence of aluminium (Sturr and Marquis 1990) and of GTF (Marquis 1995; Koo and others 2006).

1.3.3.2 Antimicrobials

Many compounds have been studied for their antimicrobial effects and are aimed at controlling dental caries by decreasing the growth of cariogenic microorganisms like *S. mutans* (Loesche 1986). Among the most common are chlorhexidine and triclosan which are broad spectrum anti-microbials. Anti-plaque agents such as chlorohexidine (bis-giguanides) are often used to reduce the mutans streptococci level (Petti and Hausen 2006). In addition, a systematic review of 22 studies did not find any conclusive evidence that chlorohexidine treatment reduces caries (Twetman 2004). Treatments with chlorohexidine have several undesirable side effects including bad taste, staining actions, and ability to react with fluoride (Featherstone 2006).

Triclosan, a phenolic compound, is commonly used in dentifrices and seemed to reduce regrowth of dental plaque (Jenkins and others 1989). It is more effective when combined with other agents such as zinc (Roberts 1995). The addition of triclosan in dentifrices did not show any benefit in toothpaste (Mellberg and others 1991; van Loveren 2000).

1.3.4 Natural products as an alternative source for novel anti-caries therapy

Natural products and their derivatives are in important source of novel structures to discover and develop new drugs for the treatment or prevention of human diseases (Koehn and Carter 2005). According to a review of the sources of new drugs over the last 25 years, 74 out 109 antibacterial New Chemical Entities are derived from natural products and their derivatives (Newman and others 2007). However, the use of natural products as a source of anti-caries therapy has not been well explored.

Our laboratory and others have identified potentially active natural molecules against cariogenic streptococci. A variety of compounds such as flavan-3-ols, flavonols, flavones, oligomeric and polymeric proanthocyanidins from cocoa, cranberry juice, cranberry, apple, and green tea have also been shown to disrupt glucans synthesis in biofilm formation (Osawa and others 2001; Ooshima 2000; Percival and others 2006; Duarte and others 2006; Koo and others 2006; Yanagida and others 2000; Hamilton-Miller 2001). Percival and others (2006) determined that a polyphenol pentamer present in cocoa reduced biofilm formation and acid production by *S. mutans*. High molecular weight polyphenolic compounds from cacao bean husk showed anti GTF activity (Osawa and others 2001; Ooshima 2000) and reduced growth rate of *S. mutans* (Ooshima 2000). The effects of cranberry juice on the glucan-mediated processes in *S. mutans* biofilm development have shown reduction in biomass, insoluble glucan, and acidogenicity (Koo and others 2006). Polyphenols from cranberry significantly disrupted accumulation and polysaccharide composition of *S.mutans* biofilms when compared to the control (Duarte and others 2006). Non-dialysable material from cranberry (NDM) and high molecular mass constituents of cranberry juice inhibited extracellular polysaccharide synthesis (Steinberg and others 2004; Yamanaka and others 2004). In a preliminary clinical trial, NDM from cranberry reduced the *S. mutans* count in saliva (Weiss and others 2002).

High molecular weight apple polyphenols inhibited the activity of GTF and the adherence of growing cariogenic bacteria (Yanagida and others 2000). Extracts of green Tea (*Camellia sinensis*), due to the presence of catechins, prevented adherence of *S. mutans* to saliva-coated hydroxyapatite, inhibited the activity of the glucosyltransferase enzyme, and had bactericidal effects against *S. mutans* (Hamilton-Miller 2001). Oolong tea polyphenols strongly inhibited enzyme activities of some type of GTases of mutans streptococci (Nakahara and others 1993) and reduced dental plaque formation in humans (Ooshima and others1994).

Polyphenols from red grapes had higher activity against *S. mutans* than fruits tested and green tea (Smullen and others 2007). Clearly, there is a need to explore alternative methods to control the onset of caries formation by using therapeutic agents aimed at the inhibition of virulence factors of *S. mutans.* Although we are focusing our research on a single, albeit important organism, we believe that our results will have importance for additional organisms present in biofilms. By aiming to disrupt the ability of *S. mutans* to utilize sucrose to form EPS, and to produce acids and acid adaptation mechanisms, therapeutic approaches to reducing the formation or virulence of cariogenic biofilms could be precise and selective, and would not necessarily suppress the resident oral flora. Considering that grape and grape pomace are widely available and harbor a myriad of substances, some of which have previously been shown to display biological activity against *S mutans*, the focus of this research was to test whether:

1.4 Hypotheses

I) Phytochemicals in grapes, especially flavonoids, can disrupt the virulence of *S. mutans* by inhibiting glucan formation and acid production;

II) The chemical composition of grapes is variable depending on variety and methods of processing, which could affect their biological activity;

III) Phytochemicals in grapes can disrupt the formation of extracellular polysaccharides matrix, which is associated with the development and cariogenicity of biofilms.

1.5 Objectives

Our objectives were to:

1) select the variety of grapes and method of processing that provide the highest biological activity on acid production by *S. mutans* and insoluble glucan synthesis.

2) determine the effects of selected varieties and processing techniques on the ability of the extracts to affect :

a) glucan synthesis

b) acid production

c) acid tolerance

4) determine the ability of the extracts to inhibit EPS matrix synthesis and biofilm formation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Grape processing

Five varieties of grapes were collected to study their phenolic composition and biological activity against *Streptococcus mutans* as follows: *Vitis vinifera* grapes, Pinot noir (PN), Cabernet Franc (CF); Vitis interspecific hybrids, Baco noir (BN), Noiret™ (NO); *Vitis labrusca*, Concord (CO). Noiret™ was a recently released wine variety selected for its deep color resulting from a cross between NY65.0467.08 and Steuben. Grapes were obtained from the 2005 harvest season from wineries and orchards across the Finger Lakes region of New York State; PN grapes from Hosmer Winery (Ovid, NY), CF grapes from Cornell Orchards (Lansing, NY), BN grapes from Pleasant Valley Winery (Hammondsport, NY), NO grapes from Swedish Hill Winery (Romulus, NY), and CO grapes from the New York State Agricultural and Experiment Station (Geneva, NY).

2.2 Grape Extracts

Powdered red grape polyphenolic extracts were prepared from whole fruit (WF), pomace (skins and seeds) after fermentation on skins (FP), after cold press (CP), and for Concord only after hot press (HP) (Figure 2.1). WF and CP samples were freeze-dried for 48 hrs, ground to powder in a PB-5A Waring blender and stored at 2 ^{o}C, protected from light. Fermentated grape pomace (FP) was washed, destemmed and crushed in a stemmer/crusher and then dispensed into 5-gal fermentation buckets. A solution of Potassium Meta Bisulfite Crystal containing 1ml 10% (w/v) per liter of grape must was added and allowed to stand for 1 hour. Diammonium Phosphate (DAP), Fermaid and yeast (Saccharomyces cerevisiae – DV10) were added at 1, 0.2 and 0.3 g/L, respectively to each bucket. Buckets were

stored at 18 ^{o}C for 9-11 days. Must was pressed when sugar level was below 0.5% as measured by an hydrometer. Juice was stored at 2 ^{o}C, until freeze-dried. Pomace was bagged and stored at -5 ^{o}C, protected from light. Hot Pressed (HP) grapes were washed, and destemmed and crushed in a stemmer/crusher. The must was heated in a steam kettle to 71 ^{o}C and cooled to 54 ^{o}C. Pectinase enzyme (0.1%) was added, the must was allowed to stand for 30 mins and then pressed in a hydraulic rack and frame press. Pomace was bagged and stored at -5 ^{o}C, protected from light.

Grape samples were frozen to –10°C and freeze dried using a Virtis SR50C freeze drier (Virtis Co., Gardiner, NY). Polyphenols were extracted from freeze-dried grapes samples as previously described (Kim and Lee 2002) with some modifications. Briefly, 200 ml of methanol/ethanol/water (50/25/25%) v/v was added to 20 g of freeze-dried sample and the mixture was sonicated in a Branson 2200 sonicator (Fisher Scienctific, Agawam, MA, U.S.A.) in ice for 20 min, and then centrifuged using a Sorvall Instruments RC5C Centrifuge (Dupont Company, Wilmington, DE, U.S.A.) at 10,000 g for 20 min. Sonication and centrifugation steps were repeated. Solvents were evaporated using a Buchi RE 121 Rotovapor (Brinkmann Instruments Inc., Westbury, NY) at 35^{o}C and reduced pressure until an aqueous polyphenolic extract was obtained. The aqueous extract was passed through preconditioned C18 Sep Pak cartridges. The cartridges were then washed with 0.01 N aqueous HCl to remove acids, sugars and other water-soluble compounds, and dried with a current of nitrogen. The polyphenols were eluted with absolute methanol. The methanol was evaporated as described above to dryness. Extracts were redissolved in distilled water and lyophilized to obtain powdered extracts.

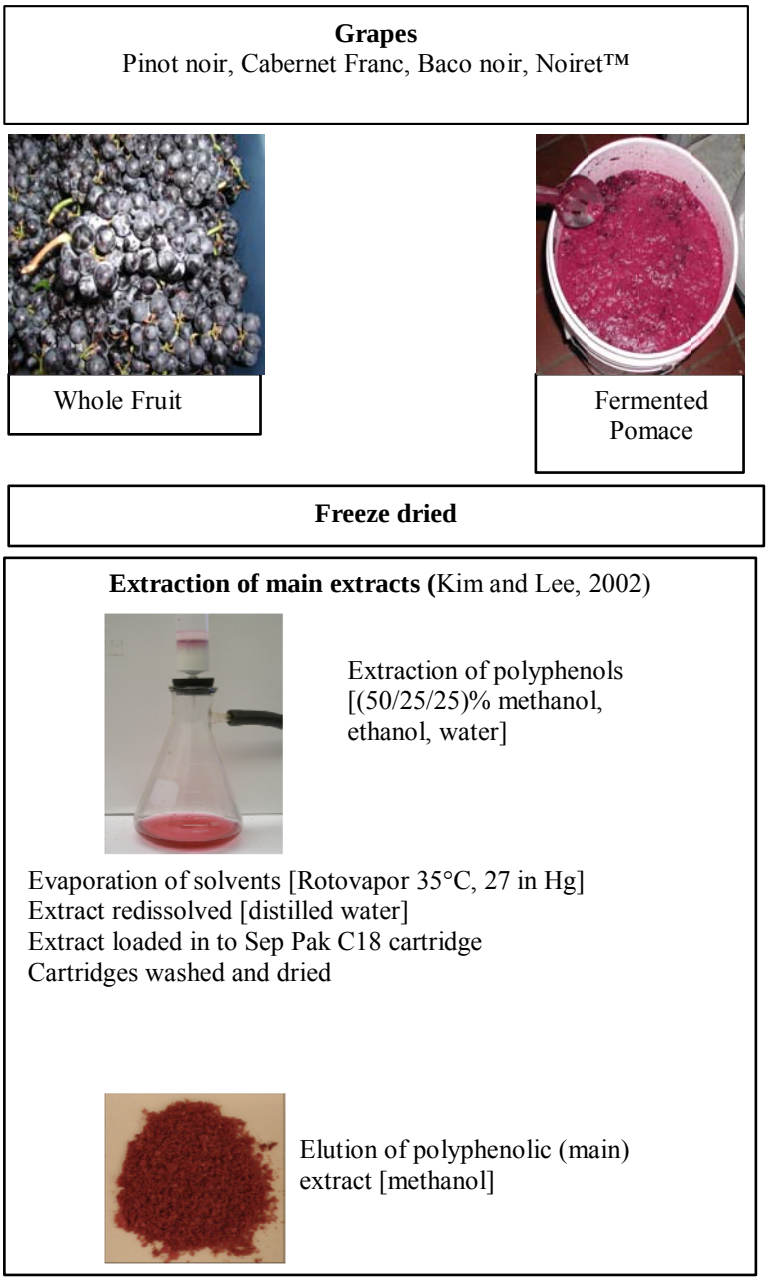


Figure 2.1. Diagram for the processing of Whole Fruit and Fermented Pomace prior to biological testing.

2.3 Determination of phenolic composition of the grape extracts

Powdered main extracts were analyzed for total phenolics, total monomeric anthocyanins, and flavan-3-ols. All analyses, per process, were performed in duplicate. Polyphenols were extracted from ground freeze-dried samples by solvent extraction with slight modifications of the method described by Kim and Lee (2002).

Total phenolic content in methanol extracts was determined by colorimetric assay using the Folin Ciocalteu (FC) reagent (Singleton and Rossi 1965), with slight modifications, and expressed as grams gallic acid equivalents (g GAE/100 g) of fruit or pomace.

The total monomeric anthocyanin content was determined using the pH differential method as described by Giusti and Wrolstad (2001) with slight modifications. Phenolic extracts were diluted in 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). After 15 min of equilibration, absorbance was measured at 520 and 700 nm using a Barnstead Turner SP830 Spectrophotometer (Barnstead International, Dubuque, Iowa, U.S.A.). The total monomeric anthocyanin content was calculated using an absorptivity coefficient and molecular weight of 28000 L/cm/mol and 529 g/mol, respectively, and expressed in g of malvidin-3-glucoside equivalents /100 g of fruit or pomace (Niketic-Aleksic and Hrazdina 1972). The total flavan-3-ol content was estimated using a modified Vanillin assay (Sun and others 1998a, 1998b, 2001). In a test tube, 2.5 ml of 1% (w/v) vanillin in methanol was added to 1 ml of appropriately diluted sample, after which 2.5 ml of 25% (v/v) H_2SO_4 in methanol was added. Absorbance was read at 500 nm, against a blank prepared in the same way except that the sample was substituted with methanol, in a Barnstead Turner SP830 Spectrophotometer (Barnstead International, Dubuque, Iowa, U.S.A.). Absorbance values were corrected for anthocyanin interference by

carrying out the reaction as described above in the absence of vanillin (Broadhurst and Jones 1978). Total flavan-3-ols were quantified using a standard curve prepared from a catechin standard obtained from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). Results were expressed as g catechin equivalents /100 g of fruit or pomace.

2.4 Determination of the biological activity of the grape extracts

2.4.1 Bacterial Strains

The bacterial strains used for the production of GTFs were: *Streptococcus anginosus* KSB8 (kindly provided by Howard K. Kuramitsu, State University of New York, Buffalo), which harbors the *gtfB* gene (for GTF B production) and *S. mutans* WHB 410 (Wunder and Bowen 1999), where *gtfB*, *gtfD* and *ftf* genes were deleted (for GTF C production) (Figure 2.2). *S. mutans* UA159, a proven virulent cariogenic pathogen and the strain selected for genomic sequencing (Adjic and others 2002) was used for F-ATPase, glycolytic pH-drop studies, antimicrobial and biofilm assays. The cultures were stored at –80º C in tryptic soy broth (TSB) containing 20% glycerol.

2.4.2 Glucosyltranferases (GTF) assays

The GTF B and C enzymes (E.C. 2.4.1.5) were prepared from culture supernatants and purified to near homogeneity by hydroxyapatite column chromatography as described previously (Venkitaraman and others 1995; Wunder and Bowen 1999). GTF B or C was mixed with a two fold dilution series of grape phenolic extracts (concentration ranging from 15.6 to 125 μg/ml) or vehicle control (10% ethanol, v/v) and incubated with [^{14}C]glucose-sucrose substrate (0.2 μCi/ml; 200 mM

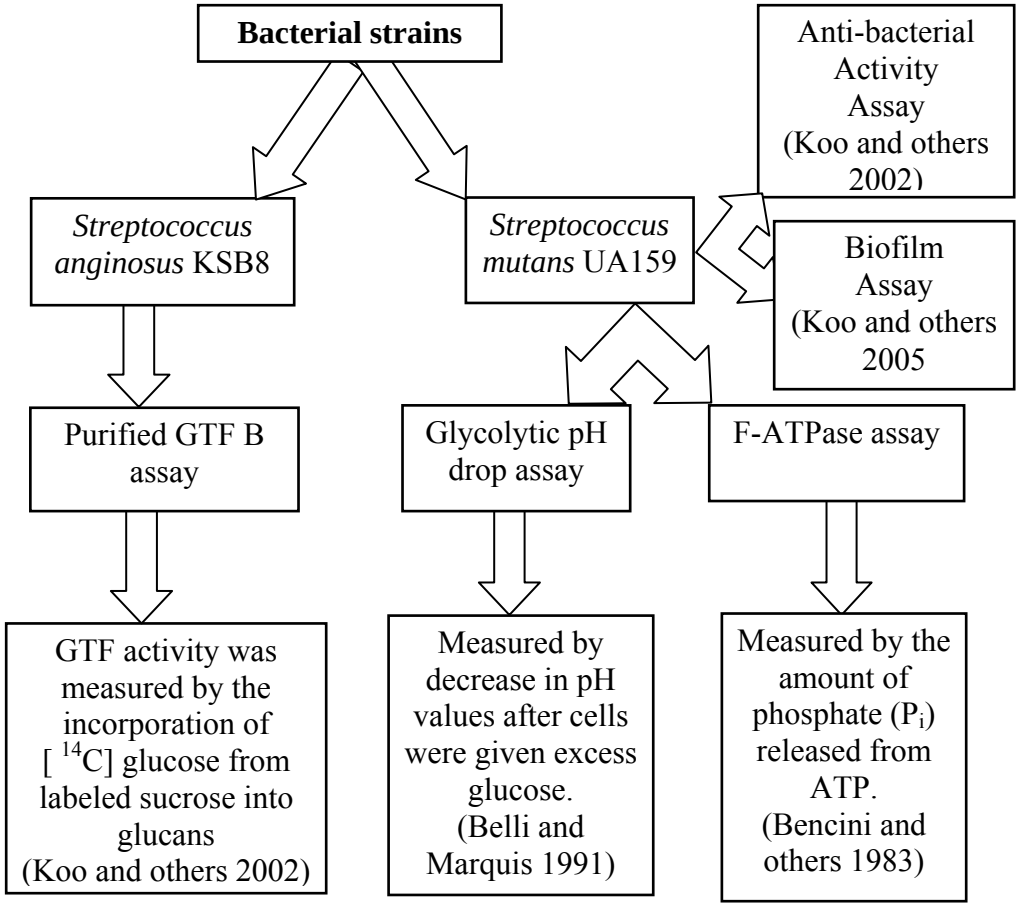


Figure 2.2 Summary diagram of biological analyses.

sucrose, 40 μM dextran 9000, and 2% sodium azide in a buffer consisting of 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, pH 6.5) to a final concentration of 100 mM sucrose (Koo and others 2002). GTF activity was measured by the incorporation of [^{14}C] glucose from labeled sucrose (NEN Research Products, Boston, MA) into glucans (Venkitaraman and others 1995; Koo and others 2000). The GTF enzyme added to each sample for all assays was equivalent to the amount required to incorporate 1 to 1.5 μmol of glucose over the 4 h reaction (1.0 – 1.5 *U*). All of the assays were conducted in triplicate from at least two separate experiments.

2.4.3 F-ATPase and glycolytic pH drop assays

F-ATPase assay was performed using permeabilized cells of *S. mutans* UA 159 (Belli and others 1995*)*. F-ATPase was measured in terms of the release of phosphate in the following reaction mixture: 75 mmol of Tris-maleate buffer (pH 7.0) containing 5 mM ATP, 10 mmol MgCl , permeabilized cells and the grape phenolic extracts (concentration ranging from 15.6 to 125 μg/ml) or vehicle control (10% ethanol, v/v). The released phosphate was determined by the method of Bencini and others (1983).

The effects of grape phenolic extracts on glycolysis were measured by standard pH drop with dense cell suspensions (Belli and others 1995). Cells of *S. mutans* UA159 were collected by centrifugation, washed with cold salt solution (50mM KCl plus 1 mM $MgCl_2$, pH 7.0), and resuspended in salt solution containing the grape phenolic extracts (concentration ranging from 62.5 to 500 μg /ml) or vehicle control (10% ethanol, v/v). Glucose was added in the mixture to give a final concentration of 1% (w/v). The decrease in pH, as a result of glycolytic activity of the bacterial cells, was assessed by means of a glass electrode (Futura Micro Combination pH electrode, 5 mm dia., Beckman Coulter, Inc., CA, USA) over a period of 75 min. (Belli and others 1995). All of the assays were conducted in triplicate from at least two separate experiments.

2.4.4 Antimicrobial assays

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each of the test compound were determined in accordance with NCCLS guidelines (NCCLS, 2000) as described in Koo and others (2003a). The broth microdilution and macrodilution methods (in TSB) were used for the antibacterial tests. The starting inoculum was 1×10^6 colony forming units/ml (at log

phase), and the concentration of test compounds ranged from 62.5 to 500 μg/mL (two-fold dilution).

2.4.5 Biofilm assays

2.4.5.1. Biofilm preparation and treatments.

Biofilms of *S. mutans* UA159 (ATCC 700610) were formed on saliva coated hydroxyapatite (sHA) discs placed in a vertical position using a disc holder (see Figure 2.3) in batch cultures at 37^{o}C and 5% CO_2 for 5 days (Koo and others 2005). The biofilms were grown in buffered tryptone yeast-extract broth containing 1% sucrose. During the first 24 hrs, the organisms were grown undisturbed to allow for initial biofilm formation. The culture medium was replaced daily; pH values in the medium were measured daily after the first 24-h of incubation. The biofilms (24 hrs old) were then treated three times daily (one-minute exposure) until the 5th day of the experimental period (126 hrs-old) with one of the following: Cab. Franc 500 μg/ml, Cab. Franc 1000 μg/ml, Baco noir 500 μg/ml, Baco noir 1000 μg/ml, or a vehicle control. Each biofilm was exposed to the respective treatment a total of twelve times.

2.4.5.2. Biofilm analyses.

At the end of the experimental period (126 h-old biofilms), the biofilms were dip-washed three times, and then gently swirled in physiological saline to remove loosely adherent material. The biofilms were placed in 5 mL of sterile saline solution, and the hydroxyapatite surfaces were gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication using three 30s pulses at output of 7 W (Branson Sonifier 150, Branson Ultrasonics, Danbury, CT,

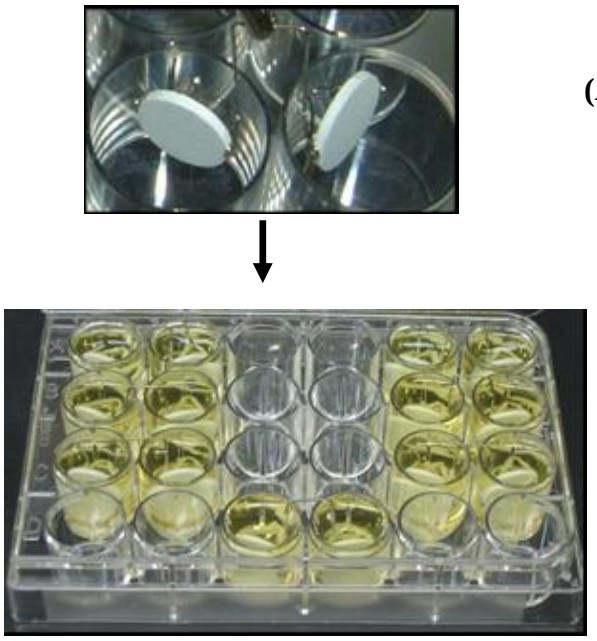


Figure 2.3. Saliva-coated hydroxyapatite (sHA) biofilm model. (A) - sHA discs placed in a vertical position; (B) – Biofilms forming in a 24-well plate.

USA). The homogenized suspension was used for dry weight, total protein, and polysaccharide analyses. For the dry weight determination, three volumes of cold ethanol (–20°C) were added to 1 mL of the biofilm suspension, and the resulting precipitate collected (10,000g for 10 min, 4°C). The supernatant was discarded, and the pellet was washed twice with cold ethanol, and then lyophilized and weighed. Total protein of the biofilm suspension was determined by acid digestion followed by ninhydrin assay (Moore and Stein 1954). The polysaccharide composition (extracellular water-soluble and insoluble, and intracellular polysaccharide) was determined by colorimetric assays as detailed by Koo and others (2003b); the

polysaccharide content was expressed per mg of dry-weight or protein. Briefly, an aliquot (4 mL) of the suspension was centrifuged at 10,000g for 10 min at 4°C. The supernatant was collected and the biofilm pellet resuspended and washed in the same volume of water; this procedure was repeated twice. All the supernatants were pooled and three volumes of cold ethanol were added, and the resulting precipitate collected. The precipitate, or water-soluble polysaccharides, were collected by centrifugation and washed three times with cold ethanol and resuspended in 1 mL of MilliQ H_2O; the total amount of carbohydrate was determined by phenol-sulphuric method (Dubois and others 1956). The biofilm pellet was dried in a Speed Vac concentrator and used for determination of: (i) extracellular insoluble polysaccharides; and (ii) intracellular iodophilic polysaccharides. Insoluble polysaccharides were extracted using 1 N NaOH (1 mg of biofilm dry weight/0.3 mL of 1 N NaOH) under agitation for 2h at 37°C. The supernatant was collected by centrifugation, and precipitated with three volumes of cold ethanol. The precipitate was washed three times with cold ethanol and resuspended in 1 mL of 1 N NaOH; the total amount of carbohydrate was determined by phenol-sulphuric method (Dubois and others 1956). Intracellular iodophilic polysaccharides were extracted with hot 5.3 M KOH (0.8 mg of biofilm dry weight/mL of KOH) and quantified using 0.2% I_2/2% KI solution as described by DiPersio and others (1974).

2.5 Statistical Analyses

Analysis of covariance (ANCOVA) was used to assess differences in percent enzyme activity between agents at each concentration. The models included categorical variables for agent, concentration, and their interaction. Overall tests were F-tests, and individual comparisons used t-tests. ANCOVA was also used to assess

differences in mean pH levels between agents at each time point. The models included categorical variables for agent, time, and their interaction. Overall tests were F-tests. Individual comparisons used t-tests. All statistical analyses were performed using SAS software (Version 9.1; SAS Institute Inc., Cary, NC). *P*-values reported are two-sided, with a level of significance set at alpha=.01.

CHAPTER 3[2]

RESULTS AND DISCUSSION

3.1 Selection of grape extracts

The phenolic content and composition in grapes can vary depending on cultivar and extraction and fermentation methods, and thereby influence the biological activity of the grape extracts. The wine grapes (Cabernet Franc, Pinot noir, Baco noir, and Noiret™) analyzed in this study were selected from our initial screening from nine different varieties of *Vitis vinifera*, *V. labrusca*, and *Vitis* interspecific hybrids. Grape berries were then studied with minimal processing into whole fruits (WF) or with extraction and fermentation methods into fermented pomace (FP).

Our initial screening was based on (i) total phenolic content, (ii) biological activity (e.g. anti GTF effect and acid production), and (iii) availability. The selected grape varieties represent important cultivars for red wine making, particularly in New York State and the northeastern United States. Figure 3.1 shows the screening and selection process for the grape extracts. The 4 grape varieties (Cab. Franc, Pinot noir, Baco noir, Noiret™) are used in red wine production, and are well-known for high phenolic content (Landrault and others 2001). Furthermore, pomace of red wine grapes is largely available as an inexpensive source of extractable material, which may present distinct chemical profile (compared to whole fruit) due to the fermentation process. In general, the extracts from two *V. vinifera* grapes showed higher total polyphenols (TP) than the non-vinifera grapes (Figure 3.2). The Concord grapes were

[2] Reproduced with permission from Thimothe J, Bonsi IA, Padilla-Zakour OI, Koo H. 2007. Chemical characterization of red wine grape (*Vitis vinifera* and Vitis interspecific hybrids) and pomace phenolic extracts and their biological activity against *Streptococcus mutans*. J. Agric. Food Chem. 55(25): 10200-10207. Copywright 2007 American Chemical Society.

not selected because their extracts were devoid of inhibitory effects against acid production by *S. mutans* as observed in our preliminary study.

3.2 Contribution of major phenolic compounds

The major phenolic compounds found in the grapes according to variety and to method of processing are summarized in Table 3.1. The total phenolic contents was variable depending on variety and wine processing method, as reported previously by others (Kammerer and others 2004; Mazza and others 1999; Macheix and others 1990*;* Netzel and others 2003). Pinot noir had the highest concentration of total phenolics and flavan-3-ols and the lowest anthocyanin content, while Noiret™ had the highest anthocyanin content. Both Noiret ™ WF and Noiret ™ FP have more than 75% of anthocyanins as their main phenolic compounds. When compared to the other varieties, Baco noir FP and Pinot noir FP had more of the flavan-3-ol monomers.

Even though pomace is a waste material from the wine fermentation process, the extracts produced from pomace for all the varieties studied had either comparable or slightly higher total phenolic content than the whole fruit extracts. Total phenols in the pomace were between 41.1 and 61.8 g of GAE/100 g of extract which was similar to that found in V. *Vinifera* var. Bangalore blue pomace (Murthy and others 2002). Furthermore, pomace extracts had similar concentrations of flavan-3-ols compared to the whole fruit, except for Baco noir, which showed twice the concentration found in the whole fruit extract. In contrast, the anthocyanin content in all of the pomace extracts was lower than that in whole fruit extracts.

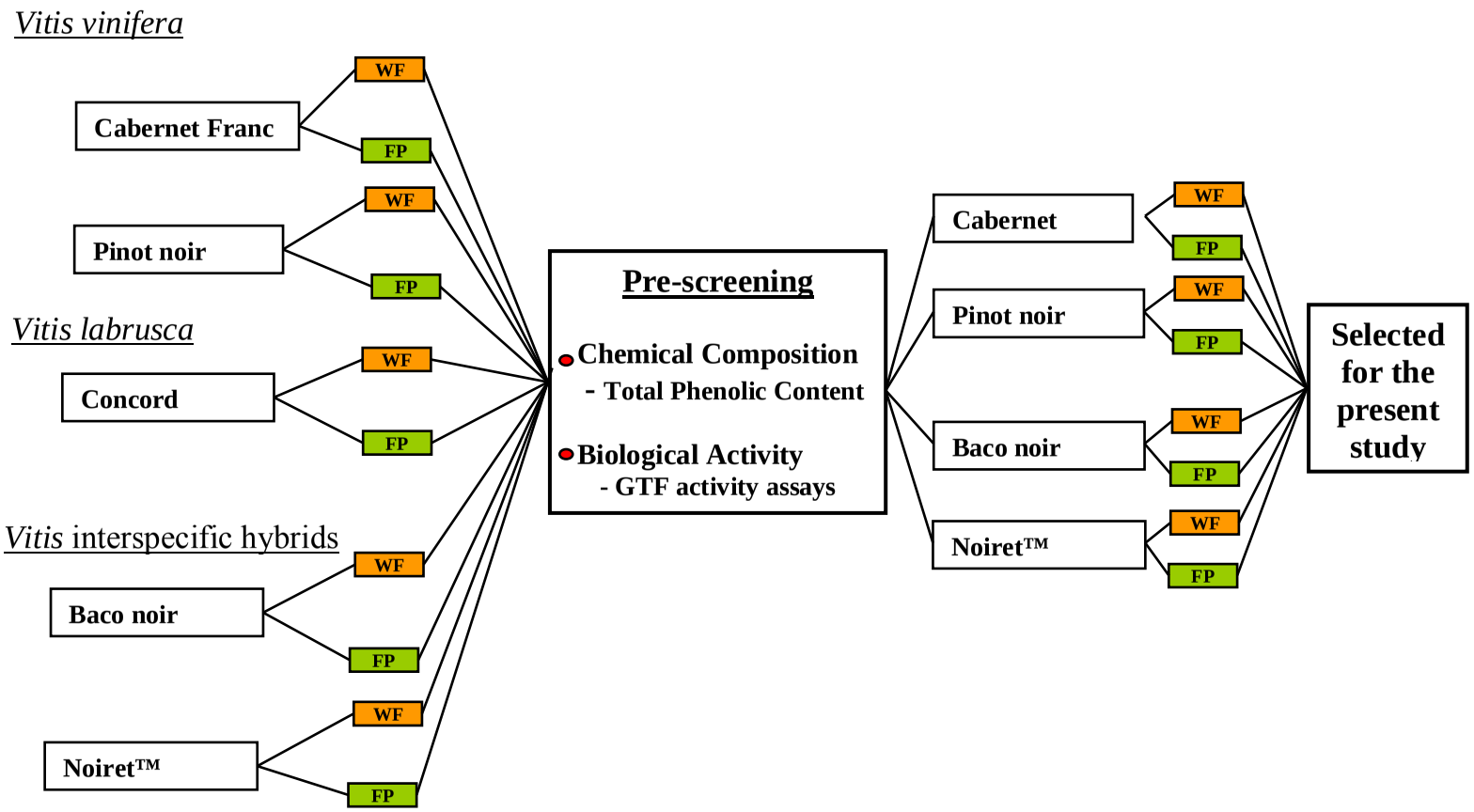


Figure 3.1. Schematic of the grape varieties and methods of processing used in this study. WF: whole fruit, FP: fermented pomace.

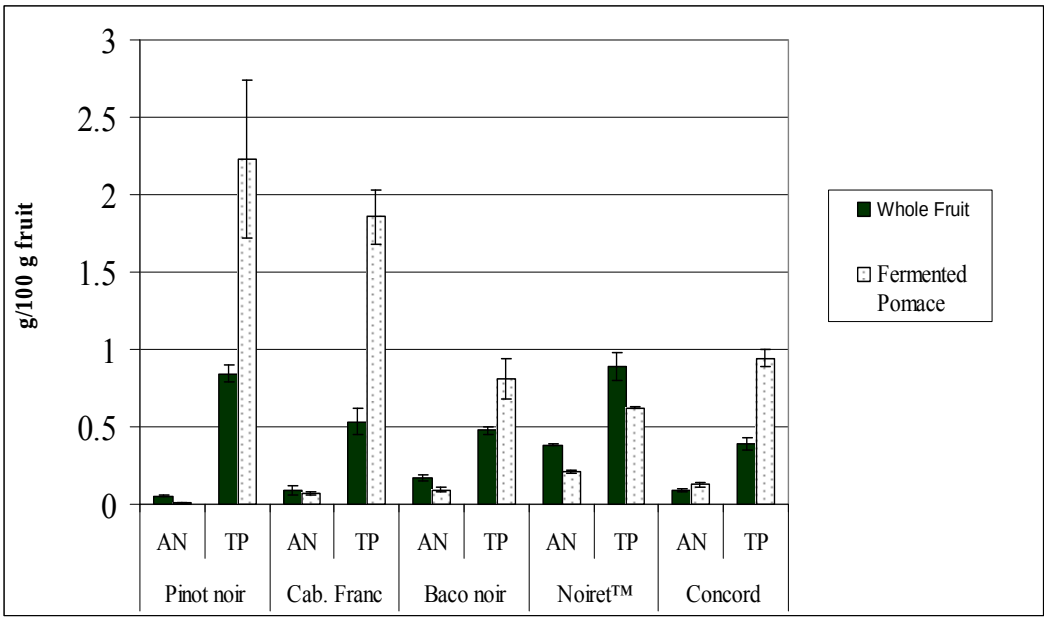


Figure 3.2. Total Phenolic and Anthocyanin content of grapes. AN: anthocyanins expressed in mg of malvidin-3-glucoside; TP: total phenols expressed in gallic acid equivalents.

3.3 Influence of grape extracts on GTF activities

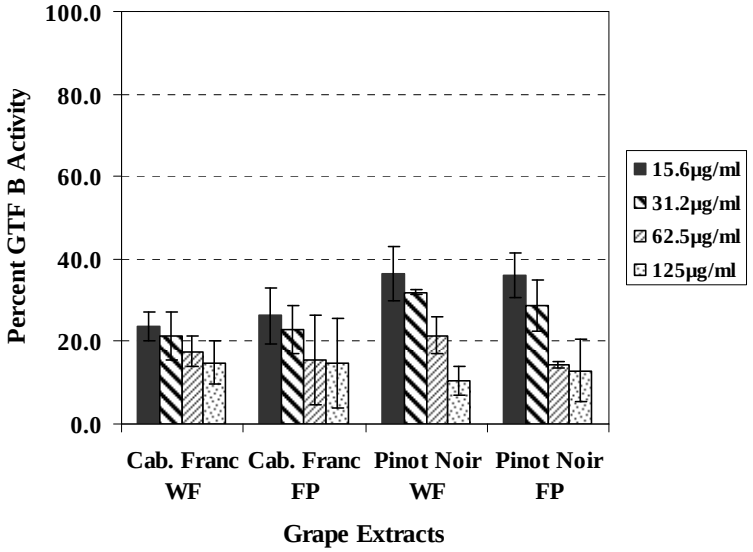
The effects of grape extracts on the activity of GTF B in solution are shown in Figure 3.3. All the grape extracts significantly reduced the activity of GTF B at all concentrations tested compared to vehicle control ($P<0.01$). The effects of grape extracts from *V. vinifera* varieties on GTF activity were similar irrespective of whether the extracts were obtained from whole fruit (WF) or pomace (FP) ($P>0.01$). The Cab Franc FP and Cab Franc WF extracts were more effective GTF inhibitors than Pinot noir extracts at concentration of 15.6 μg/ml ($P<0.01$). In contrast, fermented pomace of extracts of Baco noir and Noiret™ inhibited the glucan synthesis more effectively than the whole fruit extracts ($P<0.01$). Individual comparisons revealed that fermented pomace of Baco noir was the most effective inhibitor among the hybrid extracts. A similar inhibitory profile was observed for GTF C (data not shown).

Table 3.1. Total Phenolic, Anthocyanin, and Flavan-3-ol Content of Freeze-dried Grape Phenolic Extracts.

Sample	Flavan-3-ol content (g Cat. eq/100 g of extract)	Total anthocyanin content (g Mal eq/100 g of extract)	Total phenolic content (g of GAE/100 g of extract)
	Vitis vinifera		
Pinot noir Whole Fruit	56.7 ± 0.0	4.67 ± 0.04	56.0 ± 6.4
Pinot noir Fermented Pomace	48.4 ± 1.3	0.59 ± 0.01	61.8 ± 0.8
Cabernet Franc Whole Fruit	28.0 ± 0.5	9.78 ± 0.14	42.5 ± 1.1
Cabernet Franc Fermented Pomace	24.2 ± 1.0	6.72 ± 0.06	46.9 ± 4.1
	Vitis interspecific hybrids		
Baco noir Whole Fruit	12.3 ± 0.7	13.46 ± 0.32	34.4 ± 3.1
Baco noir Fermented Pomace	30.0 ± 0.2	3.99 ± 0.01	51.5 ± 0.4
Noiret™ Whole Fruit	19.6 ± 0.8	22.70 ± 0.20	37.1 ± 5.0
Noiret™ Fermented Pomace	18.0 ± 0.1	16.54 ± 0.26	41.1 ± 1.1

[a]Means ± standard deviation of duplicate samples. Abreviations: GAE, gallic acid equivalents; Mal Eq, malvidin-3-glucoside equivalents, Cat Eq, catechin equivalents.

A.



B.

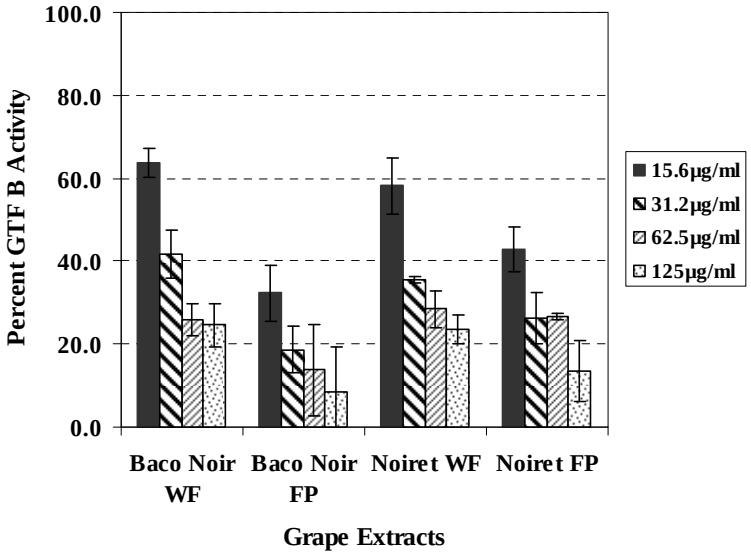


Figure 3.3. Influence of selected grape extracts a) *Vitis vinifera and* b) *Vitis* interspecific hybrids) on the activity of GTF B in solution. The final concentration of ethanol in each assay is 10% (v/v). The percent of enzyme activity is calculated against a sample with no extract as 100% GTF B activity. All of grape extracts (n=9) significantly reduced the activity of GTF B at all concentrations tested compared to vehicle control ($P<0.01$). WF:Whole Fruit; FP: Fermented Pomace.

Glucosyltransferases are specific and proven virulence traits of *S. mutans* associated with (i) pathogenesis of dental caries and (ii) bulk and structural integrity of dental Biofilm (plaque) (Bowen 2002). The glucans synthesized by these enzymes promote the binding and accumulation of *S. mutans* and other cariogenic bacteria on the tooth surface and contribute to the formation of the biofilm matrix (Bowen 2002; Schilling and Bowen 1992; Yamashita and others 1993). Therefore, one of the strategies to control biofilm formation and dental caries is to inhibit the activity of GTFs; GTF B (which synthesizes a polymer of mostly insoluble α1,3-linked glucan) and GTF C (which synthesizes a mixture of insoluble α 1,3-linked glucan and soluble α1,6-linked glucan) were used in our assays because these enzymes have been shown to be essential for the expression of virulence by *S. mutans* in causing dental caries *in vivo* (Yamashita and others 1993).

In general, the phenolic extracts from pomace were highly effective GTF inhibitors (>60% inhibition) even at concentrations as low as 15.6 μg/mL; this level of inhibition has not been observed previously (Koo and others 2006; Wunder and Bowen 1999) and would certainly disrupt the formation of cariogenic biofilms based on our *in vitro* and *in vivo* studies (Koo and others 2003a; Yamashita and others 1993). The putative bioactive compound(s) of grape phenolic extracts (especially pomace) that are modulating the GTF inhibition are unknown. Nevertheless, the presence of various flavonoids, such as low molecular weight flavonols, may be associated with enzyme inhibition. Inhibitory effect of flavonols is often associated with the presence of an unsaturated double bond between C-2 and C-3, which may provide a site for nucleophilic addition by side chains of amino acids in GTFs (Duarte and others 2006).

However, the presence of flavan-3-ols monomers and anthocyanins which lack a double bond between C-2 and C-3 exhibited either negligible or modest inhibitory activities (Yanagida and others 2000; Koo and others 2006). This observation could, in part, explain why the anti-GTF activity of the various grape extracts was not affected by major qualitative and quantitative differences in anthocyanin and flavan-3-ol content. Clearly, there are other compounds, such as high molecular weight proanthocyanidins (oligomers of flavan-3-ols), that may be involved in the GTF inhibition by the grape phenolic extracts. Further chemical analysis and research with individual compounds is needed to elucidate the specificity and the mechanistic details of GTF inhibition by the grape extracts. Although grape extracts contain weak

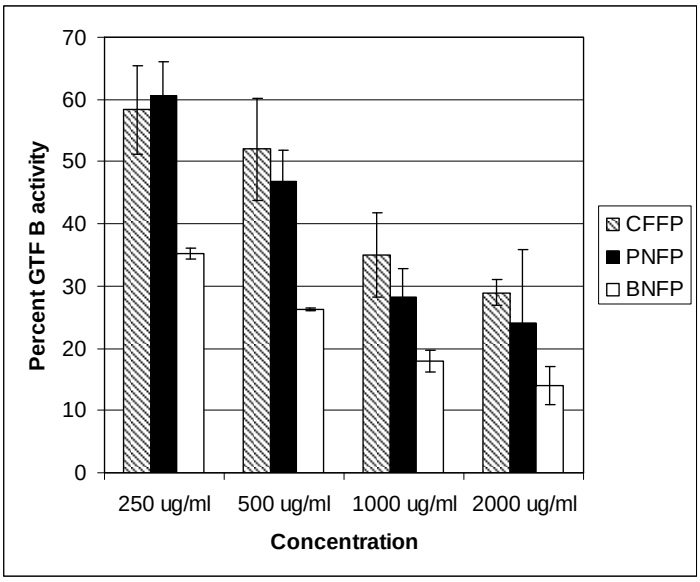


Figure 3.4. Influence of grape extracts on the activity of GTF B on surface. The final concentration of ethanol in each assay is 10% (v/v). The percent of enzyme activity is calculated against a sample with no extract as 100% GTF B activity. CFFP: Cabernet Franc fermented pomace; PNFP: Pinot noir fermented pomace; BNFP: Baco noir fermented pomace.

phenolic acids, they did not influence the pH of the reaction mixture; the pH values were between 6.8 and 6.9, which are within optimum pH for the activity of GTF enzymes (pH 6.5 and 7.0) (Schilling & Bowen 1988). Clearly, the grape extracts are highly effective against GTF enzymes, especially pomace extracts.

The fermented pomace of Cabernet Franc (CFFP), Pinot noir (PNFP), and Baco noir (BNFP) inhibited the activities of surface adsorbed GTF B by up to 80% at 4 concentrations (250, 500, 1000, and 2000μg/ml) (Figure 3.4). BNFP showed the highest inhibition when compared to the other 2 extracts. All extracts were significantly different from the control ($P<0.01$).

3.4 Influence of grape extracts on *S. mutans* acidogenicity.

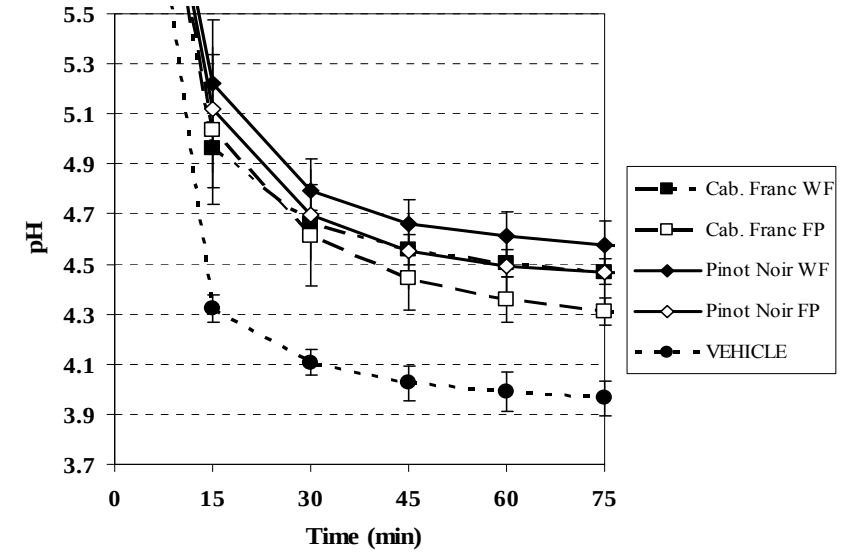
The effects of grape extracts on aciduric and acidogenic properties of *S. mutans* were examined by glycolytic pH-drop and F-ATPase activity assays. The results of pH-drop experiments in suspensions of *S. mutans* UA 159 with excess glucose in the presence of grape extracts (or vehicle control) are presented in Figure 3.5. *S. mutans* can survive and carry out glycolysis at low pH which can lead to the demineralization of the adjacent dental enamel leading to formation of carious lesions (Belli and Marquis 1991). *S. mutans* cells rapidly degrade glucose and lower the pH value of the suspension until they can no longer maintain a cytoplasmic pH compatible with activity of glycolytic enzymes. Acid sensitization can be rapidly seen in glycolytic pH-drop experiment in which cells are given excess glucose. Thus, the rate of pH drop reflects acidogenic capacities of the cells, while final pH values of the suspensions reflect acid tolerance.

All of grape extracts significantly disrupted glycolytic acid production by *S. mutans* cells ($P<0.01$) without affecting bacterial viability; however, the effects were more evident at high concentrations (500μg/ml). The presence of grape extracts sensitized the cells to acidification to the point that the final pH values (t_{75min}) were significantly higher (0.25–0.7 units) than those in the presence of vehicle control ($P < 0.01$), except Baco noir WF and Noiret WF ($P > 0.01$), indicating that acid tolerance of *S. mutans* was affected. The effects may be related to F-ATPase inhibition since the extracts were devoid of any biocidal activity (as determined by plating aliquots of cell suspension at each time point, and counting the colony forming units/mL). The proton translocating F-ATPase protects *S. mutans* against environmental acid stress by regulating pH homeostasis, which is critical for the optimum function of glycolysis (Yanagida and others 2000). Enolase and other enzymes of the glycolytic pathway and the sugar transport system are sensitive to cytoplasmic acidification (Belli and others 1995).

As shown in Figure 3.6, the F-ATPase activity of *S. mutans* UA159 was partially inhibited (23-69%) by grape extracts at 62.5 μg/ml (except Noiret™ extracts) and 125 μg/ml ($P<0.01$). All of the *V. vinifera* grapes extracts (Cabernet Franc WF, Cabernet Franc FP, Pinot noir WF, Pinot noir FP), and Baco noir FP and Noiret™ FP inhibited at least 48% of the enzyme activity at 125 μg/ml; these same extracts also showed higher final pH values in the glycolytic pH drop experiments as shown in Figure 3.5. Overall, the F-ATPase sensitivities to grape extracts agree with the pH drop data, and shows that the fermented pomace of all grape varieties tested is biologically active against *S. mutans* acidogenicity.

Flavonoids have been shown to inhibit various forms of ATPase, including *S. mutans* membrane-associated F-ATPase activity (Duarte and others 2006; Gregoire and others 2007). Among them, quercetin, myricetin, and their glycosides moderately

A.



B.

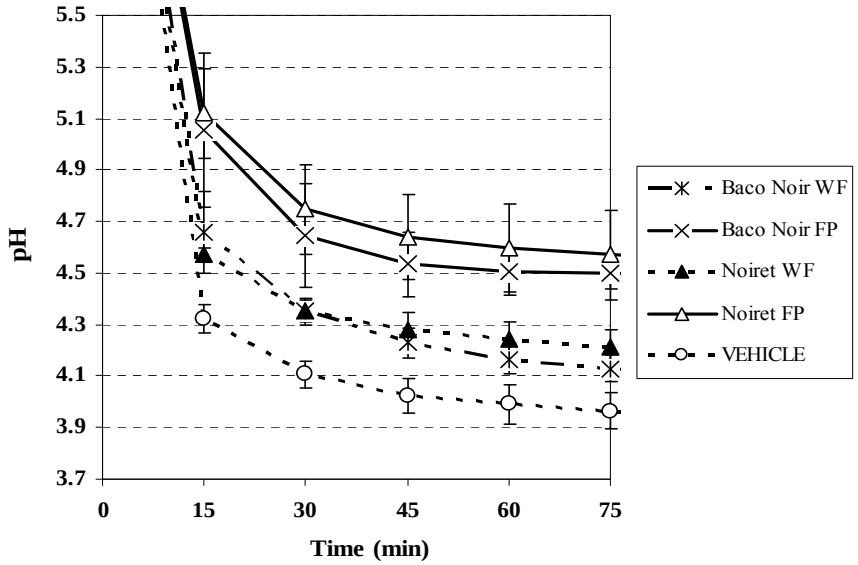
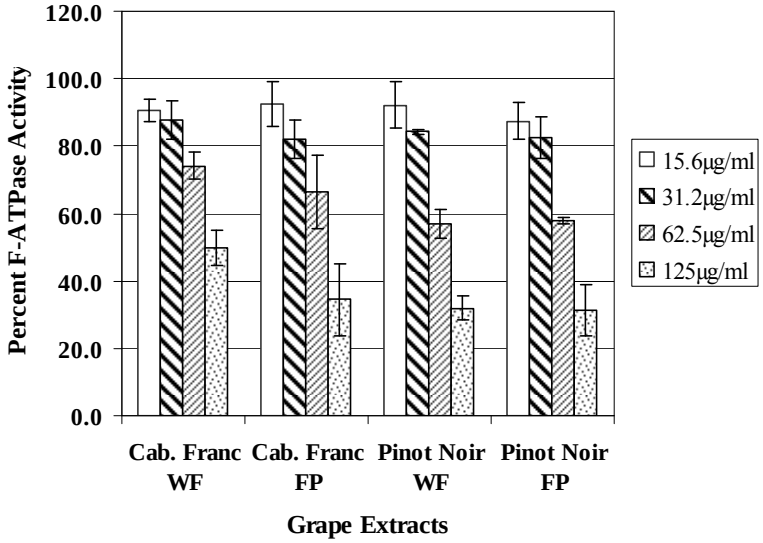


Figure 3.5. Influence of selected grape extracts a) *Vitis vinifera* and b) *Vitis* interspecific hybrids on glycolytic pH drop assay of *S. mutans* UA 159 in suspension. Planktonic cells in presence of excess glucose and of grape extracts at 500 µg/ml. Values (SD, n=6) from all of grape extracts and from vehicle control are significantly different at time points $t_{15\ min}$, $t_{30\ min}$, $t_{45\ min}$, and $t_{60\ min}$ ($P<0.01$). WF: whole fruit; FP: fermented pomace.

A.



B.

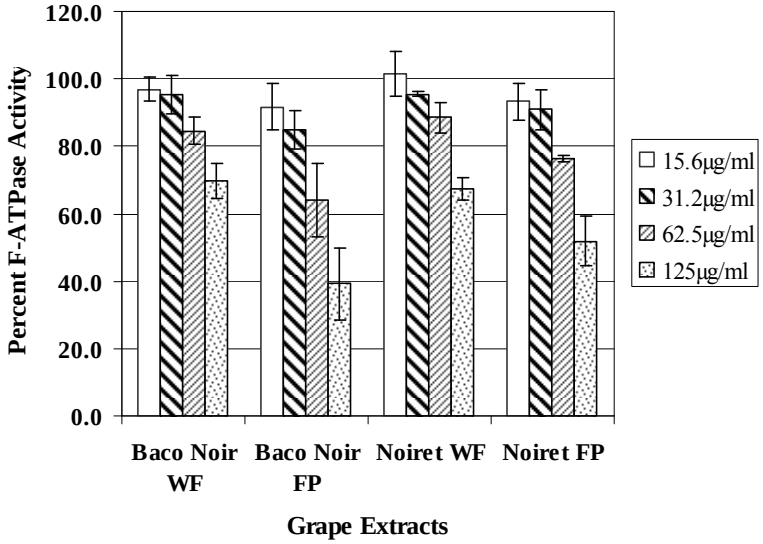


Figure 3.6. Effects of selected grape extracts a) (*Vitis vinifera*) and b) (*Vitis* interspecific hybrids) on F-ATPase activity. The final concentration of ethanol in each assay is 10% (v/v). The percent of enzyme activity is calculated against a sample with no extract as 100% F-ATPase activity. All of grape extracts (n=9) significantly reduced the activity of F-ATPase at 125 µg/ml compared to vehicle control ($P<0.01$). WF: whole fruit; FP: fermented pomace.

inhibited the activity of F-ATPases (15–35% inhibition) but only at high concentrations (150–300 μg/mL) (Gregoire and others 2007). Anthocyanins and low-molecular weight flavan-3-ols (e.g. epicatechin) were devoid of any inhibitory effects against F-ATPase (Duarte and others 2006; Gregoire and others 2007). As observed for GTF activity assays, the biological effects on acidogenicity of *S. mutans* may involve other unidentified compounds, and were not affected by the differences in the anthocyanins and flavan-3-ols content of the various grape phenolic extracts.

Interestingly, none of the extracts showed effects on the growth of *S. mutans* at concentrations tested in this study (up to 500 μg/mL), although other studies have reported selected antimicrobial activity of grape extracts against *Listeria monocytogenes* (Daglia and others 2007) and of wine extracts against *Staphylococcus aureus, Escherichia coli*, *Candida albicans*, *Streptococcus pyogenes*, and oral streptococci (Papadopoulou and others 2006; Daglia and others 2007). However, the antimicrobial activity of wine extracts against oral streptococci was mainly attributed to the presence of organic acids rather than polyphenols (Daglia and others 2007), which could explain the lack of microbiocidal activity of the grape phenolic extracts tested in this study.

Overall, grape phenolic extracts exhibit biological activity against *S. mutans* by disrupting essential virulence traits (especially GTF activity) of this ubiquitous oral pathogen involved in formation and acidogenicity of dental biofilms without affecting bacterial viability; grape extracts may harbor specific compounds that may be useful for biofilm control.

3.5 Effects of the grape extracts on polysaccharide composition in biofilms

Insoluble glucans produced by GTFs provide structural integrity and bulk to biofilms (Bowen 2002). The inhibition of GTF B activity by grape phenolic extracts indicates that the extracts may affect insoluble glucan synthesis and reduce biofilm formation. *S. mutans* in the mouth are commonly found in plaque biofilms, so we assessed the effects of short-term topical application (one-minute exposure, three times daily) on viability, biomass (dry-weight), total protein, and extracellular and intracellular polysaccharides content of *S. mutans* biofilms formed on apatic surface covered by salivary pellicle (Koo and others 2005). The regimen of one-minute exposure and daily treatments was selected for this experiment to simulate the likely exposure of test agents at clinical level. Higher levels of the agent were used for biofilms because of higher biomass densities of biofilms and previous findings that biofilms are less sensitive to grape extracts than cells in suspension.

Viability of the biofilms (as assessed by determination of colony forming units) and total protein content was not significantly impacted by topical applications of grape extracts ($P > 0.01$); likely due to the brief exposure to the agent and higher bacterial densities in biofilms. Nevertheless, short-term topical application of grape extracts disrupted the composition of the extracellular polysaccharides of *S. mutans* biofilms by reducing the content of insoluble polysaccharides (Table 3.2). The biofilms treated with the grape extracts exhibited 33.7 to 53.5% less insoluble polysaccharides than those treated with negative (vehicle) control, which agrees with the GTF data in Figure 3.3. However, only Cab. Franc and Baco noir at 1000 μg/ml reached statistical significance ($P<0.01$). In contrast, the content of extracellular water soluble and intracellular iodophilic (IPS) polysaccharides were not significantly different compared to vehicle control ($P>0.01$), except the IPS content of biofilms treated with Baco noir at 1000 μg/ml.

Interestingly, short-term exposure to the grape extracts did not significantly affect the acidogenic properties of the biofilms as indicated by the similar pH-values of the surrounding medium at various time points compared to those from vehicle-treated biofilms; only Baco noir extract at 1000 μg/ml showed some inhibitory effect.

We also determined the dry-weight of each of the treated biofilms. However, biofilms treated with grape extracts were highly pigmented indicating that compounds (probably high molecular weight tannins) in these extracts may have been incorporated in the biofilms matrix, which could adversely affect the precise determination of the dry-weight. Therefore, the dry-weight results were not used in this experiment. Although our mono-species biofilm model does not mimic the complex microbial community found in dental plaque, it does place emphasis on a critical, and perhaps the most important, part of the biofilm i.e. the polysaccharide matrix. We chose *S. mutans* as our model organism because this bacterium is a key contributor to the synthesis of EPS matrix (which are comprised mostly of glucans), and acid production within biofilms. Furthermore, biofilms using a single organism are advantageous in examining specific actions of therapeutic agents on *S. mutans* physiology, especially on the glucan-mediated process. Clearly, the grape extracts at 1000 μg/ml significantly disrupted the insoluble polysaccharide content of *S. mutans* biofilms compared with the vehicle control. The insoluble polysaccharides (comprised of mostly α1,3 and α1,6 linkages, and branch points of 3,4-, 3,6- and 3,4,6-linked glucose) are the major components of the extracellular polysaccharide matrix, which are associated with the development, bulk and cariogenicity of dental biofilms (Bowen 2002; Paes Leme and others 2006). This observation is consistent with the effective inhibition of GTF B observed in this study. *S. mutans* treated with therapeutic agents that repress the expression of GTF B, or mutant strains defective in *gtfB*, are far less

Table 3.2. Polysaccharide content in the biofilms after treatments with grape extracts.

	INS μg/mg of protein	% Inhibition	SOL μg/mg of protein	% Inhibition	IPS μg/mg of protein	% Inhibition
Vehicle Control	1.99±0.51 [a]		0.29±0.05 [a]		0.54±0.15 [a]	
Cabernet Franc 500 μg/ml	1.32±0.39 [a,b]	33.7	0.41±0.04 [a]	-	0.55±0.09 [a]	-
Cabernet Franc 1000 μg/ml	1.17±0.17 [b]	41.2	0.40±0.10 [a]	-	0.56±0.14 [a]	-
Baco noir 500 μg/ml	1.26±0.27 [a,b]	36.7	0.45±0.21 [a]	-	0.51±0.08 [a]	5.6
Baco noir 1000 μg/ml	0.93±0.22 [b]	53.3	0.32±0.12 [a]	-	0.35±0.03 [b]	35.2

Mean ± standard deviation (n = 9). Values in the same column followed by same superscripts are not significantly different from each other (P>0.01). INS: insoluble; SOL: soluble; IPS: intracellular iodophilic polysaccharide.

cariogenic than untreated or parent strains *in vivo* (Yamashita and others 1993; Koo and others 2005, 2006). Thus, disruption of insoluble glucan synthesis is one of the mechanisms by which the grape extracts may affect the development of cariogenic biofilms; further studies using our animal model of dental caries shall determine whether these extracts display cariostatic properties *in vivo*.

Overall, the data show that grape phenolic extracts are biologically active against some of the virulence traits of *S. mutans* involved in the formation of glucans and acidogenicity. However, the extracts did not affect bacterial viability neither against planktonic cell or biofilms of *S. mutans*. The putative pathways by which grape phenolic extracts affect the virulence of *S. mutans* may involve at least two routes: (1) inhibiton of insoluble glucans synthesis by GTF B and GTF C; (2) inhibition of acid tolerance and acid production mechanisms.

Furthermore, we have observed that pomace extracts were as effective, or in some cases more bioactive than the whole fruit extracts. Fermented pomace is a promising and inexpensive source for the extraction and isolation of individual polyphenols that could be used for the prevention of dental caries.

It is important to note that all the extracts tested in this study were comprised of complex mixture of different classes of compounds. Previous studies have shown that many of the constituents are either non-active or even have deleterious effects. Further fractionation and characterization could provide critical information on the potential bioactivity of individual bioactive grape components on virulence factors of *S. mutans*. The identification of these individual bioactive components used alone or in combination could further increase biological activity *in vitro.*

CHAPTER 4

Summary and future research

Grapes are a rich source of flavonoids and are widely available in the New York State area for the production of wine. Wine grapes, particularly red wine grapes, contain a wide range of polyphenolic compounds. Many studies of polyphenols have focused on their health benefits although little information is available on the prevention of dental caries. Studies of polyphenols from cranberry, cocoa, green tea, and apple reveal that many phenolic compounds inhibit virulence factors associated with one of the most cariogenic bacteria, *Streptococcus mutans*. Grapes contain many of the polyphenols seen in other natural products that inhibit glucan synthesis and acid production by *S. mutans*. However, as one of the most produced fruit crops, grapes provide a large source of raw materials for the extraction of polyphenols. Extracts from grape by-products were used to determine their influence on virulence factors associated with *S. mutans.*

Polyphenolic compounds in wine grapes contribute to color, astringency, and bitterness and have certain health benefits such as antimicrobial, anticarcinogenic, and antioxidant properties. Polyphenols are a large class of compounds that include flavonoids (anthocyanins, flavan-3-ols) and non-flavonoids. Polyphenolic compounds differ in quality and quantity depending on variety and processing method. In general, red wine grapes contain more polyphenolic compounds than white wine and table grapes.

Although grape pomace is a waste material from the processing of grapes into wine, it has been found to contain as much or higher total phenolics and flavan-3-ols

than the unpressed grape, (whole fruit). Consequently, grape pomace could be a potential source for the extraction of beneficial polyphenols.

Polyphenols have been shown to reduce the biomass of polysaccharides that form biofilms. Biofilms are communities of microbes growing on a surface and enclosed in an extracellular polyssacharide matrix also called glucans. The polysaccharides form a mesh that protect bacteria (e.g. *S. mutans*) involved in dental caries. It has been hypothesized that reducing or preventing the formation of these polysaccharides can lead to a reduction in biomass which could subsequently lead to a reduction in caries. *S. mutans* has two main virulence factors. The first one is associated with the production of glucans by enzymes such as glucosyltransferases (GTFs). The glucans synthesized by *S. mutans* help produce biofilms on tooth surfaces which are also called dental plaque. The second virulence factor allows *S. mutans* to produce acid from dietary sucrose and to survive in acidic conditions inside the biofilm. Inhibition of GTF activities is one of the strategies to control biofilm formation.

Polyphenols from natural sources such as cranberry and cocoa affect the bacteria involved in dental caries. Quercetin and proanthocyanidins significantly inhibit glucosyltransferases (GTF) and acid production mediated by F-ATPase, as shown in glycolytic pH drop experiments.

In this study, red wine grapes (Cabernet Franc, Baco noir, Pinot noir, Noiret™) were collected from several wineries across the Finger Lakes region of New York State. They were then processed and analyzed for chemical and biological activity. Grape pomace extracts were as effective as or better than the whole fruit extracts in reducing the activity of GTF and F-ATPase irrespective of the variety of the grape tested. Biological activities of pomace and fruit extracts were not dependent on the amount of anthocyanins present in the extracts which is similar to results of previous

studies. Anthocyanins were not essential for expression of biological activity. Other polyphenols from grape extracts inhibited the development of *S. mutans in vitro* by inhibiting the formation of glucans, thereby blocking bacterial adherence and preventing accumulation of *S. mutans.*

This study demonstrated that polyphenols from whole grapes or fermented pomace exhibited biological activity against *S. mutans* by disrupting essential virulence traits of this ubiquitous oral pathogen. However, the polyphenols studied did not affect bacterial viability. This indicates that grape extracts contain specific compounds that may be useful to control some important aspects in biofilm formation. Grape extracts affect virulence factors of *S. mutans* involved in the inhibition of the GTF B and C enzymes in solution and on surface, and they disrupt acid production, as shown in glycolytic pH drop assays, by inhibiting proton-translocating F-ATPase activity.

Grape extracts reduced the amount of some of the polysaccharides produced when compared to the control. In contrast, except for Baco noir at 1000 μg/ml, the content of extracellular water soluble and intracellular iodophilic polysaccharides were not significantly different from the vehicle control. Short-term exposure of the biofilms to the grape extracts did not significantly affect the viability, total protein or acidogenic properties of the biofilms. Results of the biofilm study might be due to: a) higher cells density found in biofilms compared to cells in planktonic environment, b) short exposure time in the extracts, and c) binding of large polyphenols to EPS.

The mechanisms by which grape polyphenols affect the virulence factors of *S. mutans* are not well known. Inhibition of virulence factors could be attributed to the presence of certain high molecular weight polyphenols, the presence of some flavonols, or the conformation of the flavonoid structure. The presence or absence of

double bonds on the anthocyanin molecules or the presence of sugar molecules may also affect GTF or acid production.

The presence of certain high molecular weight flavonoids such as tannins seemed to have decreased some effect on bioactivity. Tannins, by binding to the proteins from salivary pellicle might have contributed to the dry-weight from the biofilms. Other unpurified fractions such as anthocyanins might also explain lack of activities.

Subsequent research will involve the extraction of individual compounds that could be used in topical applications. Individual components of polyphenols isolated from other products have already been shown to affect biological activities. *In vitro* studies could analyze these compounds to elucidate their effects on virulence factors associated with the pathogenesis of dental plaque formation and caries. Recently published data on individual components from cranberry polyphenols have shown both individual and synergistic effects of polyphenols on virulence factors of *S. mutans*. Thus, it is recommended that individual components of grape extract polyphenols be looked at in combination as well as alone.

Other subsequent research could involve the study of the expression of *gtf* genes in biofilms. Pilot data have shown that *S. mutans* in the presence of flavonoids affects the level of expression of *gtf* genes.

Although *S. mutans* is one of the most cariogenic bacteria, other bacteria can also be involved in the caries process. Therefore, studying the effects of grape polyphenols on multispecies biofilm could lead to a better understanding of the ability of grape polyphenols to affect the biomass, acidogenicity and polysaccharide composition of biofilms in the presence of a less isolated model system.

Finally, the benefits of such research projects could only be complete by analyzing *in vivo* the effects of the extracts on *S. mutans* in dental plaque in animal

models. The goal of *in vivo* studies would be to see how effective the grape extracts are in the presence of the multi-species bioflims and complex environmental conditions that exist in the mouth. Grape seed extract has been shown to have no toxic effects in rats and could be used in topical application without toxicity concern. The approach to be used for the study would utilize frequent exposure to food and beverages and the brief application of grape components to the tooth surface. Individual components of grape extracts could affect bacterial adherence and plaque pH. Grape pomace is an inexpensive and abundant source for the extraction of individual components of polyphenols that could be used in the treatment or prevention of dental caries.

APPENDIX A

GTF B solution assay

Agents	cpm	Blank cpm	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
250ug/ml 150 uL of enzyme													
CFWF1	583	0	583	46327	2316.3335	0.252	1999	29.16	70.84	58.94	38.46	41.06	38.46
CFWF1	2046	0	2046	46327	2316.3335	0.883	1999	102.36	-2.36				
CFWF1	906	0	906	46327	2316.3335	0.391	1999	45.31	54.69				
CFWF2	592	0	592	46327	2316.3335	0.256	1999	29.64	70.36	45.01	28.39	54.99	28.39
CFWF2	552	0	552	46327	2316.3335	0.238	1999	27.62	72.38				
CFWF2	1555	0	1555	46327	2316.3335	0.671	1999	77.78	22.22				
CFCP1	510	0	510	46327	2316.3335	0.220	1999	25.53	74.47	24.94	0.92	75.06	0.92
CFCP1	477	0	477	46327	2316.3335	0.206	1999	23.89	76.11				
CFCP1	508	0	508	46327	2316.3335	0.219	1999	25.42	74.58				
CFCP2	1069	0	1069	46327	2316.3335	0.461	1999	53.47	46.53	37.99	13.92	62.01	13.92
CFCP2	680	0	680	46327	2316.3335	0.293	1999	34.00	66.00				
CFCP2	530	0	530	46327	2316.3335	0.229	1999	26.51	73.49				
CFFP1	608	0	608	46327	2316.3335	0.262	1999	30.40	69.60	40.42	20.98	59.58	20.98
CFFP1	1290	0	1290	46327	2316.3335	0.557	1999	64.53	35.47				
CFFP1	526	0	526	46327	2316.3335	0.227	1999	26.32	73.68				
CFFP2	2139	0	2139	46327	2316.3335	0.923	1999	106.99	-6.99	59.60	41.10	40.40	41.10
CFFP2	759	0	759	46327	2316.3335	0.328	1999	37.98	62.02				
CFFP2	676	0	676	46327	2316.3335	0.292	1999	33.82	66.18				
Vehicle	1491	0	1491	46327	2316.3335	0.644	1999	74.60	25.40	100.00	44.48	0.00	44.48
Vehicle	3026	0	3026	46327	2316.3335	1.306	1999	151.36	-51.36				
Vehicle	1480	0	1480	46327	2316.3335	0.639	1999	74.04	25.96				
CPM control - average			1999			0.863							

Agents	cpm	Blank cpm	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
250ug/ml													
125 uL of enzyme													
BNWF1	1385	0	1385	46327	2316.33	0.598	1999	69.30	30.70	55.31	19.49	44.69	19.49
BNWF1	1271	0	1271	46327	2316.33	0.549	1999	63.60	36.40				
BNWF1	661	0	661	46327	2316.33	0.285	1999	33.05	66.95				
BNWF2	1822	0	1822	46327	2316.33	0.786	1999	91.13	8.87	58.09	29.18	41.91	29.18
BNWF2	945	0	945	46327	2316.33	0.408	1999	47.27	52.73				
BNWF2	717	0	717	46327	2316.33	0.310	1999	35.87	64.13				
BNCP1	1927	0	1927	46327	2316.33	0.832	1999	96.38	3.62	87.98	11.33	12.02	11.33
BNCP1	1501	0	1501	46327	2316.33	0.648	1999	75.10	24.90				
BNCP1	1848	0	1848	46327	2316.33	0.798	1999	92.46	7.54				
BNCP2	1282	0	1282	46327	2316.33	0.554	1999	64.15	35.85	58.78	5.46	41.22	5.46
BNCP2	1179	0	1179	46327	2316.33	0.509	1999	58.96	41.04				
BNCP2	1064	0	1064	46327	2316.33	0.459	1999	53.24	46.76				
BNFP1	565	0	565	46327	2316.33	0.244	1999	28.29	71.71	28.42	0.70	71.58	0.70
BNFP1	583	0	583	46327	2316.33	0.252	1999	29.17	70.83				
BNFP1	556	0	556	46327	2316.33	0.240	1999	27.79	72.21				
BNFP2	638	0	638	46327	2316.33	0.276	1999	31.93	68.07	30.51	1.61	69.49	1.61
BNFP2	575	0	575	46327	2316.33	0.248	1999	28.75	71.25				
BNFP2	617	0	617	46327	2316.33	0.266	1999	30.85	69.15				
Vehicle	1491	0	1491	46327	2316.33	0.644	1999	74.60	25.40	100.00	44.48	0.00	44.48
Vehicle	3026	0	3026	46327	2316.33	1.306	1999	151.36	-51.36				
Vehicle	1480	0	1480	46327	2316.33	0.639	1999	74.04	25.96				
CPM control - average			1999			0.863							

Agents	cpm	Blank cpm	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
250ug/ml													
125 uL of enzyme													
PNWF1	1931	0	1931	46327	2316.33	0.834	1999	96.62	3.38	84.00	27.65	16.00	27.65
PNWF1	2061	0	2061	46327	2316.33	0.890	1999	103.09	-3.09				
PNWF1	1045	0	1045	46327	2316.33	0.451	1999	52.30	47.70				
PNWF2	629	0	629	46327	2316.33	0.272	1999	31.47	68.53	41.47	12.33	58.53	12.33
PNWF2	753	0	753	46327	2316.33	0.325	1999	37.69	62.31				
PNWF2	1104	0	1104	46327	2316.33	0.477	1999	55.25	44.75				
PNFP1	514	0	514	46327	2316.33	0.222	1999	25.73	74.27	26.81	2.59	73.19	2.59
PNFP1	498	0	498	46327	2316.33	0.215	1999	24.94	75.06				
PNFP1	595	0	595	46327	2316.33	0.257	1999	29.77	70.23				
PNFP2	882	0	882	46327	2316.33	0.381	1999	44.13	55.87	43.31	17.61	56.69	17.61
PNFP2	506	0	506	46327	2316.33	0.218	1999	25.31	74.69				
PNFP2	1209	0	1209	46327	2316.33	0.522	1999	60.50	39.50				
Vehicle	1491	0	1491	46327	2316.33	0.644	1999	74.60	25.40	100.00	44.48	0.00	44.48
Vehicle	3026	0	3026	46327	2316.33	1.306	1999	151.36	-51.36				
Vehicle	1480	0	1480	46327	2316.33	0.639	1999	74.04	25.96				
CPM control - average			1999			0.863							

Agents	cpm	Blank cpm	cpm-blk cpm	cpm sucrose	1 umol glucose	units	cpm control	%of activity	%of inhibition	Average %of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
250ug/ml 200 uL of enzyme													
CFWF2	444	71	373	44553	2227.67	0.168	4905	7.61	92.39	8.17	0.55	91.83	0.55
CFWF2	499	71	428	44553	2227.67	0.192	4905	8.72	91.28				
CFWF2	471	71	400	44553	2227.67	0.180	4905	8.16	91.84				
CFCP2	705	100	606	44553	2227.67	0.272	4905	12.34	87.66	14.20	3.41	85.80	3.41
CFCP2	694	100	594	44553	2227.67	0.267	4905	12.11	87.89				
CFCP2	989	100	890	44553	2227.67	0.399	4905	18.13	81.87				
CFFP2	759	121	638	44553	2227.67	0.286	4905	13.01	86.99	17.42	7.08	82.58	7.08
CFFP2	792	121	671	44553	2227.67	0.301	4905	13.67	86.33				
CFFP2	1376	121	1255	44553	2227.67	0.564	4905	25.59	74.41				
Vehicle	5019	155	4864	44553	2227.67	2.184	4905	99.16	0.84	100.00	9.25	0.00	9.25
Vehicle	5533	155	5378	44553	2227.67	2.414	4905	109.64	-9.64				
Vehicle	4629	155	4474	44553	2227.67	2.008	4905	91.20	8.80				
CPM control - average			4905			2.202							

Agents	cpm	Blank cpm	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
250ug/ml													
200 uL of enzyme													
BNWF2	1090	71	1019	44553	2227.67	0.457	6227	16.36	83.64	28.19	15.26	71.81	15.26
BNWF2	1490	71	1419	44553	2227.67	0.637	6227	22.79	77.21				
BNWF2	2899	71	2828	44553	2227.67	1.269	6227	45.41	54.59				
BNCP2	1042	100	943	44553	2227.67	0.423	6227	15.14	84.86	13.57	1.87	86.43	1.87
BNCP2	816	100	716	44553	2227.67	0.322	6227	11.50	88.50				
BNCP2	975	100	876	44553	2227.67	0.393	6227	14.06	85.94				
BNFP2	1657	121	1536	44553	2227.67	0.689	6227	24.66	75.34	16.51	7.06	83.49	7.06
BNFP2	900	121	779	44553	2227.67	0.350	6227	12.51	87.49				
BNFP2	890	121	769	44553	2227.67	0.345	6227	12.35	87.65				
Vehicle	5797	156	5642	44553	2227.67	2.533	6227	90.60	9.40	100.00	8.84	0.00	8.84
Vehicle	6890	156	6734	44553	2227.67	3.023	6227	108.13	-8.13				
Vehicle	6462	156	6307	44553	2227.67	2.831	6227	101.27	-1.27				
CPM control - average			6227			2.796							

Agents	cpm	Blank cpm	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
250ug/ml													
200 uL of enzyme													
PNWF2	1271	0	1271	44553	2227.67	0.571	6386	19.91	80.09	17.60	2.00	82.40	2.00
PNWF2	1041	0	1041	44553	2227.67	0.467	6386	16.30	83.70				
PNWF2	1060	0	1060	44553	2227.67	0.476	6386	16.59	83.41				
PNFP2	842	0	842	44553	2227.67	0.378	6386	13.19	86.81	21.59	13.75	78.41	13.75
PNFP2	902	0	902	44553	2227.67	0.405	6386	14.13	85.87				
PNFP2	2392	0	2392	44553	2227.67	1.074	6386	37.46	62.54				
Vehicle	6386	0	6386	44553	2227.67	2.867	6386	100.00	0.00	100.00	0.00	0.00	0.00
CPM control - average			6386			2.867							

APPENDIX B

GTF B in solution for Concord grapes variety.

Agents 62.50ug/ml enzyme	cpm	Blank cpm	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition
COWF1	1224	88	1136	46987	2349.33	0.483	4999	22.72	77.28	23.35	14.07	76.65
COWF1	568	88	480	46987	2349.33	0.204	4999	9.61	90.39			
COWF1	1974	88	1886	46987	2349.33	0.803	4999	37.73	62.27			
COCP1	1279	82	1197	46987	2349.33	0.510	4999	23.95	76.05	21.71	1.96	78.29
COCP1	1127	82	1046	46987	2349.33	0.445	4999	20.92	79.08			
COCP1	1095	82	1014	46987	2349.33	0.431	4999	20.27	79.73			
COFP1	1956	95	1861	46987	2349.33	0.792	4999	37.23	62.77	28.19	11.62	71.81
COFP1	1708	95	1613	46987	2349.33	0.687	4999	32.26	67.74			
COFP1	849	95	754	46987	2349.33	0.321	4999	15.09	84.91			
COHP1	1574	85	1489	46987	2349.33	0.634	4999	29.79	70.21	22.03	10.99	77.97
COHP1	1427	85	1342	46987	2349.33	0.571	4999	26.85	73.15			
COHP1	557	85	472	46987	2349.33	0.201	4999	9.45	90.55			
Vehicle	5300	156	5145	46987	2349.33	2.190	4999	102.90	-2.90	100.00	2.92	0.00
Vehicle	5008	156	4852	46987	2349.33	2.065	4999	97.06	2.94			
Vehicle	5157	156	5001	46987	2349.33	2.129	4999	100.04	-0.04			
CPM control - average			4999			2.128						

Agents 62.50ug/ml enzyme	cpm	Blank cpm	cpm - blk	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition
COWF2	1561	91	1470	45853	2292.67	0.641	3013	48.79	51.21	37.62	9.69	62.38
COWF2	1069	91	978	45853	2292.67	0.427	3013	32.46	67.54			
COWF2	1043	91	952	45853	2292.67	0.415	3013	31.60	68.40			
COCP2	958	90	868	45853	2292.67	0.379	3013	28.81	71.19	35.19	7.41	64.81
COCP2	1395	90	1305	45853	2292.67	0.569	3013	43.31	56.69			
COCP2	1098	90	1008	45853	2292.67	0.440	3013	33.46	66.54			
COFP2	847	74	773	45853	2292.67	0.337	3013	25.66	74.34	37.50	19.90	62.50
COFP2	1896	74	1822	45853	2292.67	0.795	3013	60.47	39.53			
COFP2	868	74	794	45853	2292.67	0.346	3013	26.37	73.63			
COHP2	1451	83	1368	45853	2292.67	0.597	3013	45.41	54.59	55.83	12.86	44.17
COHP2	1646	83	1563	45853	2292.67	0.682	3013	51.88	48.12			
COHP2	2198	83	2115	45853	2292.67	0.923	3013	70.20	29.80			
Vehicle	4134	156	3979	45853	2292.67	1.735	3013	132.05	-32.05	100.00	36.42	0.00
Vehicle	3396	156	3241	45853	2292.67	1.413	3013	107.56	-7.56			
Vehicle	1975	156	1820	45853	2292.67	0.794	3013	60.39	39.61			
CPM control - average			3013			1.314						

APPENDIX C

GTF B surface

Agents	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
500 ug/ml		150 uL of enzyme									
CFWF1	685	41772	2088.6	0.328	1907	35.90	64.10	37.16	2.84	62.84	2.84
CFWF1	671	41772	2088.6	0.321	1907	35.17	64.83				
CFWF1	771	41772	2088.6	0.369	1907	40.41	59.59				
CFWF2	861	41772	2088.6	0.412	1907	45.14	54.86	43.32	1.63	56.68	1.63
CFWF2	801	41772	2088.6	0.384	1907	41.99	58.01				
CFWF2	817	41772	2088.6	0.391	1907	42.83	57.17				
CFCP1	726	41772	2088.6	0.348	1907	38.08	61.92	32.57	8.60	67.43	8.60
CFCP1	705	41772	2088.6	0.338	1907	36.98	63.02				
CFCP1	432	41772	2088.6	0.207	1907	22.67	77.33				
CFCP2	617	41772	2088.6	0.295	1907	32.35	67.65	38.29	5.95	61.71	5.95
CFCP2	730	41772	2088.6	0.350	1907	38.27	61.73				
CFCP2	844	41772	2088.6	0.404	1907	44.25	55.75				
CFFP1	627	41772	2088.6	0.300	1907	32.86	67.14	39.37	5.95	60.63	5.95
CFFP1	850	41772	2088.6	0.407	1907	44.56	55.44				
CFFP1	776	41772	2088.6	0.371	1907	40.68	59.32				
CFFP2	665	41772	2088.6	0.318	1907	34.86	65.14	36.59	1.76	63.41	1.76
CFFP2	697	41772	2088.6	0.334	1907	36.54	63.46				
CFFP2	732	41772	2088.6	0.350	1907	38.38	61.62				
Vehicle	1892	41772	2088.6	0.906	1907	99.18	0.82	100.00	7.97	0.00	7.97
Vehicle	1764	41772	2088.6	0.844	1907	92.47	7.53				
Vehicle	2067	41772	2088.6	0.990	1907	108.35	-8.35				
CPM control - average	1907			0.913							

Agents	cpm - blk cpm	cpm sucrose	1 umol glucose	units	cpm control	% of activity	% of inhibition	Average % of activity	Stdev % activity	Average % inhibition	Stdev % inhibition
500 ug/ml		750 uL of enzyme									
CFWF1	509	43072	2153.6	0.236	1553	32.75	67.25	36.14	5.87	63.86	5.87
CFWF1	667	43072	2153.6	0.310	1553	42.92	57.08				
CFWF1	509	43072	2153.6	0.236	1553	32.75	67.25				
CFWF2	711	43072	2153.6	0.330	1553	45.77	54.23	40.32	18.63	59.68	18.63
CFWF2	304	43072	2153.6	0.141	1553	19.57	80.43				
CFWF2	864	43072	2153.6	0.401	1553	55.62	44.38				
CFCP1	567	43072	2153.6	0.263	1553	36.52	63.48	30.34	6.31	69.66	6.31
CFCP1	475	43072	2153.6	0.221	1553	30.60	69.40				
CFCP1	371	43072	2153.6	0.172	1553	23.90	76.10				
CFCP2	373	43072	2153.6	0.173	1553	24.01	75.99	26.37	3.14	73.63	3.14
CFCP2	391	43072	2153.6	0.182	1553	25.17	74.83				
CFCP2	465	43072	2153.6	0.216	1553	29.93	70.07				
CFFP1	640	43072	2153.6	0.297	1553	41.19	58.81	38.55	6.32	61.45	6.32
CFFP1	670	43072	2153.6	0.311	1553	43.12	56.88				
CFFP1	487	43072	2153.6	0.226	1553	31.34	68.66				
CFFP2	323	43072	2153.6	0.150	1553	20.79	79.21	23.78	2.67	76.22	2.67
CFFP2	403	43072	2153.6	0.187	1553	25.94	74.06				
CFFP2	382	43072	2153.6	0.177	1553	24.59	75.41				
Vehicle	1356	43072	2153.6	0.630	1553	87.28	12.72	100.00	32.53	0.00	32.53
Vehicle	2128	43072	2153.6	0.988	1553	136.97	-36.97				
Vehicle	1177	43072	2153.6	0.546	1553	75.75	24.25				
CPM control - average	1553			0.721							

APPENDIX D

F-ATPase data for Cabernet Franc, Pinot noir, and Baco noir extracts under different conditions.

500 ug/ml, 15% EtOH	Conc ug/ml		conc. difference ug/ml	% activity	% inhibition
	test	test neg			
CFWF1	1.89	1.70	0.19	8.75	91.25
CFWF2	1.96	1.83	0.14	6.19	93.81
Water	3.52	1.31	2.21		
Water	3.52	1.31	2.21		
Al & F	2.50	1.67	0.83	37.45	62.55

15% EtOH, 500 ug/ml, 1% innoculum	Conc ug/ml		conc. difference ug/ml	% activity	% inhibition
	test	test neg			
CFWF1	2.11	1.99	0.12	4.92	95.08
CFCP1	2.23	2.17	0.06	2.43	97.57
Vehicle	3.95	1.52	2.42		
CFFP1	2.14	2.04	0.10	4.24	95.76
Vehicle	4.03	1.57	2.46		
Water	3.94	1.54	2.40		
Al & F	3.01	2.15	0.86	35.90	64.10

15% EtOH, 500 ug/ml, 1% innoculum	Conc ug/ml		conc. difference ug/ml	% activity	% inhibition
	test	test neg			
CFWF2	2.53	2.45	0.09	4.80	95.20
CFCP2	2.51	2.54	-0.03	-1.71	101.71
Vehicle	3.65	1.85	1.80		
CFFP2	2.24	2.70	-0.46	-25.78	125.78
Vehicle	3.67	1.88	1.79		
Water	4.08	1.94	2.14		
Al & F	3.14	2.63	0.51	23.93	76.07

15% EtOH, 500 ug/ml, 1% innoculum	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFWF2	2.63	2.66	-0.03	-0.99	100.99
CFCP2	2.34	2.59	-0.25	-9.45	109.45
Vehicle	4.56	1.93	2.64		
Water	4.67	2.02	2.65		
Al & F	3.40	2.71	0.70	26.24	73.76
	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFFP2	2.61	2.48	0.13	5.05	94.95
Vehicle	4.59	1.98	2.60		
Water	4.67	2.02	2.65		
Al & F	3.40	2.71	0.70	26.24	73.76

15% EtOH, 500 ug/ml, 1% innoculum	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFWF1	2.46	2.25	0.21	12.70	87.30
CFCP1	2.48	2.50	-0.02	-1.30	101.30
Vehicle	3.41	1.73	1.68		
Water	4.44	1.83	2.61		
Al & F	3.28	2.51	0.77	29.47	70.53
	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFFP1	2.41	2.32	0.09	5.47	94.53
Vehicle	3.41	1.85	1.57		
Water	4.44	1.83	2.61		
Al & F	3.28	2.51	0.77	29.47	70.53

125 ug/ml, 10% EtOH 2nd incubation 5 min	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFWF1 5 MIN	2.98	1.74	1.24	36.47	63.53
Vehicle 5 MIN	5.42	2.03	3.39		
Water 5 MIN	5.06	2.23	2.84		
Al & F 5 MIN	3.80	2.35	1.45	51.21	48.79
125 ug/ml 2nd incubation 15 min	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFWF1 15 MIN	2.86	1.86	1.00	33.01	66.99
Vehicle	4.95	1.93	3.02		

5 min for 2nd incubation					
125ug/ml, 10% EtOH	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFWF1	2.93	1.48	1.45	63.76	36.24
CFCP1	2.25	1.51	0.74	32.43	67.57
CFFP1	2.72	1.49	1.23	54.32	45.68
Vehicle	3.75	1.49	2.27		
Water	4.37	1.43	2.94		
Al & F	3.44	2.18	1.26	42.81	57.19

5 min for 2nd incubation					
125ug/ml, 10% EtOH	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
BNWF1	2.41	1.29	1.12	76.67	23.33
BNCP1	2.06	1.32	0.75	51.18	48.82
BNFP1	1.95	1.34	0.61	41.55	58.45
Vehicle	2.71	1.24	1.46		
Water	3.48	1.21	2.26		
Al & F	2.90	2.08	0.82	36.11	63.89

10% EtOH	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
BNCP1 125 ug/ml	2.58	1.77	0.81	52.18	47.82
BNCP1 250 ug/ml	2.37	1.92	0.45	29.18	70.82
BNCP1 500 ug/ml	2.29	2.40	-0.11	-7.17	107.17
Vehicle	3.23	1.69	1.55		
Water	4.02	1.65	2.38		
Al & F	3.42	2.49	0.93	38.90	61.10

10% EtOH	Conc ug/ml test	test neg	conc. difference ug/ml	% activity	% inhibition
CFCP1 125 ug/ml	2.37	1.84	0.54	23.67	76.33
CFCP1 250 ug/ml	2.08	1.94	0.14	6.17	93.83
CFCP1 500 ug/ml	2.32	2.42	-0.10	-4.49	104.49
Vehicle	4.02	1.75	2.27		
Water	3.90	1.74	2.16		
Al & F	3.27	2.49	0.78	36.07	63.93

	Conc ug/ml		conc. difference ug/ml	% activity	% inhibition
	test	test neg			
BNWF1 250 ug/ml	3.11	1.96	1.16	45.80	54.20
BNCP1 250 ug/ml	2.46	2.02	0.44	17.56	82.44
BNFP1 250 ug/ml	2.35	1.94	0.41	16.13	83.87
Vehicle	4.23	1.70	2.53		
Water	4.48	1.73	2.75		
Al & F	3.63	2.45	1.18	42.81	57.19

	Conc ug/ml		conc. difference ug/ml	% activity	% inhibition
	test	test neg			
PNWF1 250 ug/ml	1.88	1.86	0.02	0.87	99.13
PNFP1 250 ug/ml	1.88	1.74	0.14	5.52	94.48
BNWF1 250 ug/ml	2.60	1.83	0.77	29.41	70.59
Vehicle	4.27	1.66	2.61		
Water	4.15	1.60	2.55		
Al & F	3.39	2.21	1.17	46.02	53.98

REFERENCES

Aires CP, Tabchoury CP, Del Bel Cury AA, Koo H, Cury JA. 2006. Effect of sucrose concentration on dental biofilm formed in situ and on enamel demineralization. Caries Res. 40(1):28-32.

Ajdic D, McShan WM, McLaughlin RE, Savic G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Quian Y, Li S, Zhu H, Najar F, Lai H, White J, Roe BA, Ferretti JJ. 2002. Genome sequence of *Streptococcus mutans* UA 159, a cariogenic dental pathogen. Proc. Natl Acad Sci. USA. 99:14434 - 14439.

Alonso-Borbalán AM, Zorro L, Guillén DA, García Barroso, C. 2003. Study of the polyphenol content of red and white grape varieties by liquid chromatography-mass spectrometry and its relationship to antioxidant power. J. of Chrom A. 1012:31-38.

Amerine A, Joslyn MA. 1967. Composition of grapes. In: Table Wines, The Technology of Their Production. 2nd ed Chapter 6. University of California Press, Berkeley, Los Angeles and London pp 234-238.

Banas JA, Vickerman MM. 2003. Glucan-binding proteins of the oral streptococci. Crit. Rev. Oral Biol. Med. 14(2):89-99.

Basha SM, Musingo M, Colova VS. 2004. Compositional differences in the phenolics compounds of muscadine and bunch grape wines. African Journal of Biotechnology. 3(10):523-528.

Beighton D. 2005. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent. Oral. Epidemiol. 33(4): 248-255.

Belli WA, Marquis RE. 1991. Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. Appl. Environ. Microbiol. 57:1134-1138.

Belli WA , Buckley DH, Marquis RE. 1995. Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5. Can. J. Microbiol. 41: 785-791.

Bencini DA, Shanley MS, Wild JR, O'Donovan GA. 1983. New assay for enzymatic phosphate release: application to aspartate transcarbamylase and other enzymes. Anal Biochem. 132: 259-264.

Bender GR, Sutton SVW, Marquis RE. 1986. Acid tolerance, proton permeabilities and membrane ATPases of oral streptococci. Infect. Immun. 53:331-338.

Blanco JL. 1998. La plus vieille histoire du vin. In L'Histoire du vin, une histoire de rites. Office International de la Vigne et du Vin. pp. 31-41.

Blasi CD, Tanzi V, Lanzette M. 1997. A study on the production of agricultural residues in Italy. Biomass and Bioenergy. 12:321-333.

Boulton IC, Cooke JA, Johnson MS. 1995. Fluoride accumulation and toxicity in laboratory populations of wild small mammals and white mice. J. Appl. Toxicol. 15(6): 423-431.

Bowen WH, Amsbaugh SM, Monell-Torrens S, Brunelle J, Kuzmiak-Jones H, Cole MF. 1980. A method to assess cariogenic potential of foodstuffs. J. Am. Dent. Assoc. 100:677-681.

Bowen WH. 1999. Wither or whither caries research? Caries Res. 33(1): 1-3.

Bowen WH. 2002. Do we need to be concerned about dental caries in the coming millennium? Crit. Rev. Oral Biol. Med. 13:126-131.

Braga FG, Lencart e Silva FA, Alves A. 2002. Recovery of winery by-products in the Douro Demarcated region: production of calcium tartrate and grape pigments. Am. J. Enol. Vitic. 53:41-45.

Brambilla E. 2001. Fluoride – Is It Capable of Fighting Old and New Dental Diseases? Caries Res. 35(suppl 1):6-9.

Broadhurst RB, Jones WT. 1978. Analysis of condensed tannin using acidified vanillin. J. Sci. Food Agric. 29:788-794.

Burns J, Gardner, PT, O'Neil J, Crawford S, Morecroft I, McPhail DB, Lister C, Matthews D, MacLean MR, Lean MEJ, Duthie GG, Crozier A. 2000. Relationship among Antioxidant Activity, Vasodilation Capacity, and Phenolic Content of Red Wines. J. Agric. Food Chem. 48: 220-230.

Burns J, Mullen W, Landrault N, Teissedre PL, Lean MEJ, Crozier A. 2002. Variations in the profile and content of anthocyanins in wines made from Cabernet Sauvignon and Hybrid grapes. J. Agric. Food Chem. 50:4096-4102.

Cantos E, Espín JC, Tomás-Barberán FA. 2002. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. J Agric Food Chem. 50(20):5691-5696.

Chamkha M, Cathala B, Cheynier V, Douillard R. 2003. Phenolic composition of champagnes from Chardonnay and Pinot noir vintages. J Agric Food Chem. 51(10):3179-3184.

Cheynier VF, Rigaud J. 1986. HPLC separation and characterization of flavonoids in the skins of *Vitis vinifera* var. Cinsault. Am. J. Enol. Viti. 37(4):248-252.

Cheynier V, Silva JMR. 1991. Oxidation of grape procyanidins in model solutions containing *trans*-caffeoyltartaric acid and polyphenol oxidase. J. Agric. Food Chem. 39: 1047–1049.

Cheynier V, Moutounet M, Sarni-Manchado P. 1998. Les composés phénoliques. In: C. Flanzy, Editor, Œnologie: fondements scientifiques et technologiques, Lavoisier Tec & Doc, Paris pp. 124–164.

Clarkson BH, Wefel JS, Feagin FF. 1986. Fluoride distribution in enamel after in vitro caries-like lesion formation. J. Dent. Res. 65(7):963-966.

Critchley P. 1969. The breakdown of the carbohydrate and protein matrix of dental plaque. Caries Res. 3:249-265.

Cury JA, Rebello MA, Del Bel Cury AA. 1997. *In situ* relationship between sucrose exposure and the composition of dental plaque. Caries Res. 31 :356-360.

Cury JA, Rebelo MA, Del Bel Cury AA, Derbyshire MT, Tabchoury CP. 2000. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res. 34:491.497.

Daglia M, Papetti A, Grisoli P, Aceti C, Dacarro C, Gazzani G. 2007. Antibacterial Activity of Red and White Wine against Oral Streptococci. J. Agric. Food Chem. 55: 5038-5042.

Daroch F, Hoeneisen M, González CL , Kawaguchi F, Salgado F, Solar H, García A . 2001. *In vitro* antibacterial activity of Chilean red wines against *Helicobacter pylori*. Microbios. 104(408):79-85.

De Candolle AP. 1886. Origin of cultivated plants (reprint 1964). New York:Hafner publishing.

Dipersio JR, Mattingly SJ, Higgins ML, Shockman GD. 1974. Measurement of intracellular iodophilic polysaccharide in two cariogenic strains of Streptococcus mutans by cytochemical and chemical methods. Infect. and Imm. 10(3):597-604.

Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ , Rice-Evans C, Roberfroid M, Stahl W, Viña-Ribes J . 1998. Functional food science and defence against reactive oxidative species. Br J Nutr. 80 Suppl 1:S77-112.

Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, Koo H. 2006. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. FEMS Microbiol. Lett. 257:50-56.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

El-Shami SM, El-Mallah MH, Mohamed SS. 1992. Studies on the lipid constituents of grape seeds recovered from pomace resulting from white grape processing. Grasas Aceites. 43: 157-160.

Emilson CG, Nilsson B, Bowen WH. 1984. Carbohydrate composition of dental plaque from primates with irradiation caries. J. Oral Path. 13:213-220.

Falchi M, Bertelli A, Lo Scalzo R , Morassut M, Morelli R , Das S, Cui J , Das DK. 2006. Comparison of cardioprotective abilities between the flesh and skin of grapes. J Agric Food Chem 54(18):6613-22.

FAOSTAT-FAO statistical database. http://www.fao.org.

Featherstone JD. 2006. Delivery challenges for fluoride, chlorhexidine and xylitol. BMC Oral Health. 6 Suppl 1:S8.

Firestone AR, Schmid R, Mühlmann HR. 1982. Cariogenic effects of cooked wheat starch alone or with sucrose and frequency-controlled feeding in rats. Arch. Oral Biol 27:759-763.

Flamini R. 2003. Mass spectrometry in grape and wine chemistry. Part I: polyphenols. *Mass* Spectrom. Rev. 22:218-250.

Fuleki T, Ricardo-Da-Silva JM. 1997. Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. J. Agric. Food Chem. 45:1156-1160.

Fuleki T, Ricardo-Da-Silva JM. 2003. Effects of cultivar and processing method on the contents of catechins and procyanidins in grape juice. J. Agric. Food Chem. 51:640-6.

Gagné S, Saucier C, Gény L. 2006. Composition and Cellular Localization of Tannins in Cabernet Sauvignon Skins during Growth. J. Agric. Food Chem. 54:9465-9471

Galet P. 2000. Dictionnaire Encyclopédique des Cépages. Hachette.

Giusti MM, Wrölstad RE. 2001. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In: Current protocols in food analytical chemistry. John Wiley and Sons, Inc.: New York, NY. 2001, F1.2.1-F1.2.13.

Glories Y. 1988. Anthocyanins and tannins from wine: organoleptic properties. Prog Clin Biol Res.280:123-34.

González-Paramás AM, Esteban-Ruano S, Santos-Buelga C, de Pascual-Teresa S, Rivas-Gonzalo JC. 2004. Flavanol content and antioxidant activity in winery by products. J Agric Food Chem 52(2):234-238.

Gregoire S, Singh AP, Vorsa N, Koo H. 2007. Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *Streptococcus mutans* acidogenicity. J. Appl. Microbiol. 103(5):1960-1968.

Hamada S, Slade HD. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev. 44(2):331-384.

Hamilton-Miller JM. 2001. Anti-cariogenic properties of tea (Camellia sinensis). J Med Microbiol. 50(4):299-302.

Hanada N, Kuramitsu HK. 1988. Isolation and characterization of the *Streptococcus mutans gtf*C gene, coding for synthesis of both soluble and insoluble glucans. Infect. Immun. 56(8):1999-2005.

Hang YD, Woodams EE. 1986. Utilization of grape pomace for citric acid production by solid state fermentation. Am. J. Enol. Vitic. 37(2): 141-142.

Hang YD, Woodams EE. 2008. Methanol content of grappa made from New York grape pomace. Bioresource Technology 99:3923-3925.

Haslam E. 1980. Oligomeric procyanidins and the aging of red wines. Phytochemistry. 19:1577-1582.

Hotz P, Guggenheim B, Schmid, R. 1972. Carbohydrates in pooled dental plaque. Caries Res. 6:103-121.

Holt RL 1975. Studies on human dental plaque. 3. Variations of protein constituents among individuals. J. Oral Path. 4:96-100.

Houde V, Grenier D, Chandad F. 2006. Protective effects of grape seed proanthocyanidins against oxidative stress induced by lipopolysaccharide of periodontopathogens. J. Periodontol. 77(8):1371-1379.

Jackson DI, Lombard PB. 1993. Environmental and management practices affecting grape composition and wine quality: A review. Am. J. Enol. Vitic. 44:409-430.

Jayaprakasha GK , Singh RP, Sakariah KK. 2001. Antioxidant activity of grape seed (*Vitis vinifera)* extracts on peroxidation models *in vitro*. Food Chemistry 73: 285-290.

Jenkins S, Addy M, Newcombe R. 1989. Toothpastes containing 0.3% and 0.5% triclosan. I. Effects on 4-day plaque regrowth. Am. J. Dent. 2 Spec:211-214.

Ju ZY, Howard LR. 2003. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. J Agric Food Chem.51(18):5207-5213.

Kallithraka S, Tsoutsouras E, Tzourou E, Lanaridis P. 2006. Principal phenolic compounds in Greek red wines. Food Chemistry 99:784-793.

Kammerer D, Claus A, Carle R, Schieber A. 2004. Polyphenol screening of pomace from red and white grape varieties (*Vitis vinifera L.*) by HPLC-DAD-MS/MS. J. Agric. Food Chem. 52:4360-4367.

Kim DO, Lee CY. 2002. Extraction and isolation of polyphenolics. In Current Protocols in Food Analytical Chemistry, John Wiley and Sons, Inc .: New York, NY. I1.2.1-I1.2.12.

Koehn FE, Carter GT. 2005. The evolving role of natural products in drug discovery. Nature Reviews. 4:206-220.

Koo H, Vacca-Smith AM, Bowen WH, Rosalen PL, Cury JA, Park YK. 2000. Effects of *Apis mellifera* propolis on the activities of streptococcal glucosyltransferases in solution and adsorbed onto saliva-coated hydroxyapatite. Caries Res. 34:361-442.

Koo H, Rosalen PL, Cury JA, Park YK, Bowen WH. 2002. Effects of compounds found in propolis on *S. mutans* growth and on glucosyltransferase activity. Antimicrob. Agents Chemother. 46:1302-1309.

Koo H, Pearson SK, Scott-Anne K, Abranches J, Cury JA, Rosalen PL, Park YK, Marquis RE, Bowen WH. 2003a. Effects of *apigenin* and *tt-farnesol* on glucosyltransferase activity, biofilm viability and caries development in rat. Oral Microbiol. Immunol. 17:337-343.

Koo H, Hayacibara MF, Schobel BD, Cury JA, Rosalen PL, Park YK, Vacca-Smith AM, Bowen WH. 2003b. Inhibition of *Streptococcus mutans* biofilms accumulation and polysaccharide production by *apigenin* and *tt-farnesol*. Journal of Antimicrob. Chemother. 52:782-789.

Koo H, Sheng J, Nguyen PTM, Marquis RE. 2005. Co-operative inhibition by fluoride and zinc of glucosyl transferase production and polysaccharide synthesis by mutans streptococci in suspension cultures and biofilms. FEMS Microbiol. Lett. 254:134-140.

Koo H, Nino de Guzman P, Schobe BD, Vacca-Smith AV, Bowen WH. 2006. Influence of cranberry juice on glucan-mediated processes involved in *Streptococcus mutans* biofilm development. Caries Res. 40:20-27.

Koyama K, Goto-Yamamoto N, Hashizume K. 2007. Influence of maceration temperature in red wine vinification on extraction of phenolics from berry

skins and seeds of grape (*Vitis vinifera*). Biosci. Biotechnol. Biochem. 71(4): 958-965.

Kuramitsu HK. 2003. Molecular genetic analysis of the virulence of oral bacterial pathogens: an historical perspective. Crit. Rev. Oral Biol. Med. 14(5):331-344.

Labarbe B, Cheynier V, Brossaud F, Souquet JM, Moutounet M. 1999. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. J. Agric. Food Chem. 47 :2719-2723.

Landrault N, Poucheret P, Ravel P, Gasc F, Cros G, Teissedre PL. 2001. Antioxidant capacities and phenolics levels of french wines from different varieties and vintages. J. Agric. Food Chem. 49:3341-3348.

Lee CY, Jaworski AW. Identification of some phenolics in white grapes. 1990. Am. J. Enol. Vitic. 41:87-89.

Lewis K. 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. 45:999-1007.

Loesche W J. 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev. 50:353-380.

Lu Y, Foo LY. 1999. The polyphenol constituents of grape pomace. Food Chem.65:1-8.

MacGregor EA, Jespersen HM, Svensson B. 1996. A circularly permuted alpha-amylase-type alpha/beta-barrel structure in glucan-synthesizing glucosyltransferases. FEBS Lett. 378:263-266.

Macheix JJ, Fleuriet A, Billot J. 1990. *Fruit Phenolics*. Boca Raton, FL: CRC Press.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. 2004. Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79:727-747.

Manios T. 2004. The composting potential of different organic solid wastes: experience from the island of Crete. Environment International. 29:1079-1089.

Marquis RE. 1990. Diminished acid tolerance of plaque bacteria caused by fluoride. J. Dent. Res. 69(Special issue): 672-675.

Marquis RE. 1995. Antimicrobial actions of fluoride for oral bacteria. Can. J. Microbiol. 41:955-964.

Marquis RE, Clock SA, Molta-Meira M. 2003. Fluoride and organic weak acids as modulators of microbial physiology. FEMS Microbiology Reviews 26:493-510.

Marsh PD. 2003. Are dental diseases examples of ecological catastrophes? Microbiology 149(2):279-294.

Mattos-Graner RO, Smith DJ, King WF, Mayer MP. 2000. Water-insoluble glucan synthesis by mutans streptococci strains correlates with caries incidence in 12-30 month old children. J Dent Res 79:1371-1377.

Mazza G, Miniati E. 1993. Anthocyanins in fruits, vegetables and grains. Boca Raton, FL: CRC Press.

Mazza G. 1995. Anthocyanins in grapes and grape products. Crit Rev Food Sci Nutr 35:341-371.

Mazza G, Fukumoto L, Delaquis P, Girard B, Ewert B. 1999. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot noir wines from British Columbia. J. Agric. Food Chem. 47:4009-4017.

McCabe RM, Donkersloot JA. 1977. Adherence of *Veillonella* species mediated by extracellular glucosyltransferase from *Streptococcus salivarius*. Infect. Inmmun. 18:726-734.

McGovern PE. 2004. Ancient wine: the search for the origins of viniculture. Princeton University Press.

Mellberg JR, Blake-Haskins J, Petrou ID, Grote NE. 1991. Remineralization and demineralization in situ from a triclosan/co-polymer/fluoride dentifrice. J Dent Res. 70(11):1441-1443.

Moore S, Stein WH. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211:907-913.

Murthy KNC, Singh RP, Jayaprakasha GK. 2002. Antioxidant activity of grape (*Vitis vinifera*) pomace extracts. J. Agric. Food Chem. 50:5909-5914.

Nakahara K, Kawabata S, Ono H, Ogura K, Tanaka T, Ooshima T, Hamada S. 1993. Inhibitory effect of Oolong tea polyphenols on glucosyltransferases of Mutans Streptococci. Applied and Env. Microbiol. 59(4):968-973.

National Institutes of Health (NIH). 2001. Diagnosis and management of dental caries throughout life. NIH Consensus Statement 18(1): 1-30.

National Agricultural Statistics Service (NASS). 2006. New York fruit tree and vineyard survey. www.nass.usda.gov.

National Agricultural Statistics Service (NASS). 2008. Fruit report July. www.nass.usda.gov.

Netzel M, Strass G, Bitsch I, Konitz R, Christmann M, Bitsch R. 2003. Effect of grape processing on selected antioxidant phenolics in red wine. J. Food Eng. 56:223-228.

Newman DJ, Cragg GM. 2007. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70: 461-477.

Niketic-Aleksic GK, Hrazdina G. 1972. Quantitative analysis of the anthocyanin content in grape juices and wines. Lebesm.-Wiss. U. Technol. 5:163-165.

Noble AC. 1990. Bitterness and astringency in wine. In:Rouseff RL editor. Developments in Food Science 25. Bitterness in foods and beverages. New York: Elsevier. p145-158.

Nobre dos Santos M, Melo dos Santos L, Francisco SB, Cury JA. 2002 Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. Caries Res. 36(5):347-352.

Nurgel C, Canbas A. 1998. Production of tartaric acid from pomace of some Anatolian grape cultivars. Am. J. Enol. Vitic. 49:95-99.

Ooshima T, Osaka Y, Sadaki H, Osawa K, Yasuda H, Matsumura M, Sobue S, Matsumoto M. 2000. Caries inhibitory activity of cacao bean husk extract in *in-vitro* and animal experiments. Archives of Oral Biology 45: 639-645.

Osawa K, Miyazaki K, Shimura S, Okuda J, Matsumoto M, Ooshima T. 2001. Identification of cariostatic substances in the cacao bean husk: Their anti-glucosyltransferase and antibacterial activities. J. Dent. Res. 80:2000-2004.

Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. 2006. The role of sucrose in cariogenic dental biofilm formation – new insight. J Dent Res 85(10): 878-887.

Papadopoulou C, Soulti K, Roussis JG. 2005. Potential antimicrobial activity of red and white wine phenolic extracts against strains of *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Food Technol. Biotechnol. 43:41-46.

Peña-Niera A, Duenas M, Duarte A, Hernandez T, Estrella I, Loyola E. 2004. Effects of ripening stages and plant vegetative vigor on the phenolic composition of grapes (*Vitis vinifera* L.) cv. Cabernet Sauvignon in the Maipo Valley (Chile). *Vitis*. 43:51-57.

Percival RS, Devine DA, Duggal MS, Chartron S, Marsh PD. 2006. The effect of cocoa polyphenols on the growth, metabolism, and biofilm formation by

Streptococcus mutans and *Streptococcus sanguinis*. Eur J. Oral Sci. 114:343-348.

Pérez-Magariño S, González-San José ML. 2004. Polyphenols and colour variability of red wines made from grapes harvested at different ripeness grade. Food Chemistry 96:197-208.

Petti S, H Hausen. 2006. Caries-preventive effect of chlorhexidine gel applications among high-risk children. Caries Res. 40:514-521.

Pollefeys P, Bousquet J. 2003. Molecular genetic diversity of the French–American grapevine hybrids cultivated in North America. Genome 46: 1037–1048.

Porter LJ. 1986. Flavans and proanthocyanidins. In: Harbone J.B., editor. The Flavonoids. Chapman and Hall, London, pp23-55.

Prieur C, Rigaud J, Cheynier V, Moutounet M. 1994. Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry. 36 (3):781-784.

Reisch BI, Pool RM, Peterson DV, Martens MH, Henick Kling T. 1993a. Wine and juice grape varieties for cool climates. Information Bulletin 233. Cornell Cooperative Extension. http://www.nysaes.cornell.edu/hort/faculty/reisch/bulletin/wine/

Reisch BI, Peterson DV, Pool RM, Martens MH. 1993b. Table grape varieties for cool climates. Information Bulletin 234. Cornell Cooperative Extension.

Revilla E, Ryan JM, Martín-Ortega G. 1995. Comparison of several procedures used for the extraction of anthocyanins from red grapes. J. Agric. Food Chem. 46: 4592-4597.

Rhodes PL, Mitchell JW, Wilson MW, Melton LD. 2006. Antilisterial activity of grape juice and grape extracts derived from *Vitis vinifera* variety Ribier. Int. J. Food Microbiol. 107:281-286.

Ribereau-Gàyon P.1982. The anthocyanins of grapes and wines. In: Markakis P, editor. Anthocyanins as food colors. Orlando, FL: Academic Press. p209-243

Ricardo-da Silva JM, Cheynier V, Souquet JM, Moutounet M, Cabanis JC, Bourzeix M. 1991. Interaction of grape seed procyanidins with various proteins in relation to wine fining. J. Sci. Food Agric. 57:111-125.

Roberts AJ. 1995. Role of models in assessing new agents for caries prevention – non-fluoride systems. Adv. Dent. Res. 9(3):304-311.

Robichaud JL, Noble, AC. 1990. Astringency and bitterness of selected phenolics in wines. J. Sci. Food Agric. 53:343-353.

Rölla G, Ciardi JE, Eggen K and others .1983. Free glucosyl- and fructosyltransferase in human saliva and adsorption of these enzymes to teeth *in vivo*. In:Doyle RJ and Ciardi JE, editors. Glucosyltransferases, Glucans, Sucrose, and Dental Caries. Washington DC, USA: Chemical Senses IRL Press p 21-30.

Rozen R, Steinberg D, Bachrach G. 2004. Streptococcus mutans fructosyltransferase interactions with glucans. FEMS Microbiol Lett. 232(1):39-43.

Russell RRB. 1994. The application of molecular genetics to the microbiology of dental caries. Caries Res 28:69-82.

Sarni-Manchado P, Cheynier V, Moutounet M. 1999. Interactions of grape seed tannins with salivary proteins. J. Agric. Food Chem. 47:42-47.

Schieber A, Stintzing FC, Carle R. 2001. By-products of plant food processing as a source of functional compounds – recent developments. Trends Food Sci. Technol. 12:401-413.

Schilling KM, Bowen WH. 1988. The activity of glucosyltransfersases adsorbed onto saliva-coated hydroxyapatite. J. Dent Res. 67:2-8.

Schilling K M, Bowen WH. 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. Infect. Imm. 60:284-295.

Sheiham A.1991.Why free sugars consumption should be below 15 kg per person per year in industrialised countries: the dental evidence. Br Dent J. 171(2):63-65.

Shi J, Yu J, Pohorly JE, Kakuda Y. 2003. Polyphenolics in grape seeds – biochemistry and functionality. J. Med. Food 6(4): 291-299.

Shiroza T, Ueda S, Kuramitsu HK. 1987. Sequence analysis of the *glucosyltransferase* B gene from *Streptococcus mutans*. J. Bacteriol 169:4263-4270.

Sims CA. 1994. Importance and control of phenols in wines. In: Dharmadhikari, M.; Edson C., Wolker, K., editors: *Proceedings of the Ninth Annual Midwest Regional Grape and Wine Conference.* Osage Beach, Missouri. Jan 30-Feb 1: 91-102.

Singleton VL, Rossi JA. 1965 Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. Am. J. Enol. Vitic. 16:144-158.

Singleton VL, Salques M, Zaya J, Trousdale E. 1985. Caftaric acid disappearance and conversion to products of enzymatic oxidation in grape must and wine. Am. J. Enol. Vitic. 36:50-56.

Singleton VL. 1992. Tannins and the qualities of wines. In: Hemingway RW, Laks PE, editors. Plant polyphenols: Synthesis, properties, significance. New York: Plenum Press. p859-880.

Smullen J, Koutsou GA, Foster HA, Zumbé A, Storey DM. 2007. The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*. J. Agric. Food Chem. 55(8):3148-3157.

Souquet JM, Cheynier V, Brossaud F, Moutonet M. 1996. Polymeric proanthocyanidins from grape skin. Phytochemistry. 43:509-512.

Souquet JM, B Labarbe, Le Guernevé C, Cheynier V, Moutounet M. 2000. Phenolic composition of grape stems. J. Agric. Food Chem. 49: 1076-1080.

Spanos GA, Wrolstad RE. 1990. Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. J. Agric. Food Chem. 38:1565-1571.

Stager LE. 1985. First fruits of civilization. In: Tubb JN, editor. Palestine in the Bronze and Iron Age. Papers in Honor of Olga Tufnell. London Institute of Archeology. p 172-187.

Stagos D, Kazantzoglou G, Theofanidou D, Kakalopoulou G, Magiatis P, Mitaku S, Kouretas D. 2006. Activity of grape extracts from Greek varieties of *Vitis vinifera* against mutagenicity induced by bleomycin and hydrogen peroxide in *Salmonella typhimurium* strain TA102. Mutation research-genetic toxicology and environmental mutagenesis 609(2):165-175.

Steinberg D., Feldman M, Ofek I, Weisss EI. 2004. Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. J. Antimicrob. Chemother. 54:86-89.

Stephan RM. 1944. Intra-oral hydrogen-ion concentrations associated with dental caries activity. J. Dent. Res. 23 :257-266.

Sturr M., Marquis RE. 1990. Inhibition of proton-translocating ATPases of *Streptococcus mutans* and *Lactobacillus casei* by fluoride and aluminum. Arch. Microbiol. 155:22-27.

Sturr MG, Marquis RE. 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. Appl. Environ. Microbiol. 58:2287-2291.

Su CT, Singleton VL. 1969. Identifications of three flavan-3-ols from grapes. Phytochemistry 8:1553-1558.

Sun B, Leandro C, Ricardo-da-Silva J. M, Spranger I. 1998a. Separation of Grape and Wine Proanthocyanidins According to their Degree of Polymerization. J. Agric. Food Chem. 46:1390-1396.

Sun B, Ricardo-da-Silva JM, Spranger I. 1998b. Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. J. Agric. Food Chem. 46:4267-4274.

Sun B, Spranger I, Roque-do-Vale F, Leandro C, Belchior P. 2001. Effect of Different Winemaking Technologies on Phenolic Composition in Tinta Miuda Red Wines. J. Agric. Food Chem. 49:5809-5816.

Tanzer JM, Freedman ML, Fitzgerald RJ 1985. Virulence of mutants defective in glucosyltransferase, dextran-mediated aggregation, or dextranase activity. In: Mergenhagen SE, Rosan B, editors. Molecular Basis of Oral Microbial Adhesion. American Society of Microbiology, Washington, DC pp. 204–211.

This P, Lacombe T, Thomas MR. 2006. Historical origins and genetic diversity of wine grapes. Trends in Genetics. 22(9): 511-519.

Torres JL, Bobet R. 2001. New Flavanol Derivatives from Grape (*Vitis vinifera*) Byproducts. Antioxidant Aminoethylthio-Flavan-3-ol Conjugates from a Polymeric Waste Fraction Used as a Source of Flavanols. J. Agric. Food Chem. 49: 4627-4634.

Torres JL, Varela B, García MT, Carilla J, Matito C, Centelles JJ, Cascante M, Sort X, Bobet R. 2002. Valorization of grape (V*itis vinifera*) byproducts. antioxidant and biological properties of polyphenolic fractions differing in procyanidin composition and flavonol content. J. Agric. Food Chem. 50: 7548-7555.

Twetman S. 2004. Antimicrobials in future caries control? Caries Res. 38:223-229.

Ueda S, Shiroza T, Kuramitsu HK. 1988. Sequence analysis of the glucosyltransferase C gene from *Streptococcus mutans* GS-5. Gene 69:101-109.

US department of health services. 2000. Oral health in America: A report of the Surgeon General – Executive Summary. Rockville, MD: U.S. Department of Health and Human Services, NICDR, NIH.

Vacca-Smith AM, Bowen WH. 1998. Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. Archives of Oral Biology 43:103-110.

Vacca-Smith AM, Bowen WH. 2000. In situ studies of pellicle formation on hydroxyapatite discs. Arch. Oral Biol. 45:277-291.

Van Loveren C, Buijs JF, TenCate JM. 2000. The effect of triclosan toothpaste on enamel demineralization in a bacterial demineralization model. J. Antimicrob. Chemotherapy 45:153-158.

Van Loveren C. 2001. Antimicrobial activity of fluoride and its *in vivo* importance: identification of research questions. 35(Suppl 1): 65-70.

Venkitaraman AR, Vacca-Smith AM, Kopec LK, Bowen WH. 1995. Characterization of glucosyltransferase B, GtfC, and GtfD in solution and on the surface of hydroxyapatite. J. Dent. Res. 74:1695-1701.

Vinson JA, Hontz BA. 1995. Phenol antioxidant index: comparative antioxidant effectiveness of red and white wines. J. Agric. Food Chem. 43:401-403.

Waterhouse AL. 2002. Wine phenolics. Ann. NY Acad. Sci. 957:21-36.

Weiss EL, Lev-Dor R, Sharon N, Ofek I. 2002. Inhibitory effects of a high-molecular-weight constituent of cranberry on adhesion of oral bacteria. Crit. Rev. Food Sci. Nutr. 42:285-292.

Wen A, Delaquis P, Stanich K, Toivonen P. 2003. Antilisterial activity of selected phenolic acids. Food Microbiology 20: 305-311.

Wunder D, Bowen WH. 1999. Action of agents on glucosyltransferases from *Streptococcus mutans* in solution and adsorbed to experimental pellicle. *Arch. Oral Biol.* 44:203-214.

Xavier JB, Picioreanu C, Rani SA, Van Loosdrecht MCM, Stewart PS. 2005. Biofilm-control strategies based on enzymic disruption of the extracellular polymeric substance matrix – a modeling study. Microbiology. 151:3817-3832.

Yamanaka A, Kimizuka R, Kato T, Okuda K. 2004. Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation. Oral Microbiol Immunol 19:150-154.

Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK. 1993. Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model. Infect. Immun. 61:3811-3817.

Yanagida A, Kanda T, Tanabe M, Matsudaira F, Oliveira Cordeiro JG. 2000. Inhibitory effects of apple polyphenols and related compounds on cariogenic factors of *S. mutans* streptococci. J Agric Food Chem 48:5666-5671.

Zafrilla PJ, Morillas J, Mulero J, Cayuela JM, Martínez-Cachá A, Pardo F, López Nicolás JM. 2003. Changes during Storage in Conventional and Ecological Wine: Phenolic Content and Antioxidant Activity. J. Agric. Food Chem. 51: 4694-4700.

Zhao J, Wang J, Chen Y, Garwall RA. 1999. Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin B5-3'-gallate as the most effective antioxidant constituent. Carcinogenesis 20(9) 1737-1745.

Zohary D, Hofp M. 1993. Domestication of plants in the old world. Oxford Clarendon Press p143-150.